JOURNAL OF BACTERIOLOGY, June 1999, p. 3382–3391 Vol. 181, No. 11
0021-9193/99/$04.00+0

Metabolic Imbalance and Sporulation in an Isocitrate Dehydrogenase Mutant of *Bacillus subtilis*

KIYOSHI MATSUNO,[1]† TESSA BLAIS,[2] ALISA W. SERIO,[1] TYRRELL CONWAY,[2] TINA M. HENKIN,[2] AND ABRAHAM L. SONENSHEIN[1]*

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111,[1] and Department of Microbiology, Ohio State University, Columbus, Ohio 43210[2]

Received 25 January 1999/Accepted 30 March 1999

A *Bacillus subtilis* mutant with a deletion in the *citC* gene, encoding isocitrate dehydrogenase, the third enzyme of the tricarboxylic acid branch of the Krebs cycle, exhibited reduced growth yield in broth medium and had greatly reduced ability to sporulate compared to the wild type due to a block at stage I, i.e., a failure to form the polar division septum. In early stationary phase, mutant cells accumulated intracellular and extracellular concentrations of citrate and isocitrate that were at least 15-fold higher than in wild-type cells. The growth and sporulation defects of the mutant could be partially bypassed by deletion of the major citrate synthase gene (*citZ*), by raising the pH of the medium, or by supplementation of the medium with certain divalent cations, suggesting that abnormal accumulation of citrate affects survival of stationary-phase cells and sporulation by lowering extracellular pH and chelating metal ions. While these genetic and environmental alterations were not sufficient to allow the majority of the mutant cell population to pass the stage I block (lack of asymmetric septum formation), introduction of the *sof-1* mutant form of the Spo0A transcription factor, when coupled with a reduction in citrate synthesis, restored sporulation gene expression and spore formation nearly to wild-type levels. Thus, the primary factor inhibiting sporulation in a *citC* mutant is abnormally high accumulation of citrate, but relief of this metabolic defect is not by itself sufficient to restore competence for sporulation.

Nutritional limitation leads *Bacillus subtilis* cells to cease normal cell division and initiate a highly organized developmental program, culminating in production of a dormant, environmentally resistant spore (11, 39). During the initiation of sporulation, cells undergo a modified cell division (asymmetric septation) in which a septum forms near one of the cell poles, creating a small forespore compartment and a larger mother cell compartment. This compartmentalization of sporulating cells restricts the activities of sporulation-specific sigma factors, causing each compartment to have a different program of gene expression (48). The ordered sequence of sporulation events is strictly governed by regulatory networks which coordinate nutritional, transcriptional, and morphological signals (47, 48). One aspect of physiological control of these networks is the metabolic and signalling role of the Krebs citric acid cycle. A strong linkage between Krebs cycle function and sporulation is reflected by the fact that Krebs cycle enzymes are maximally induced just before the onset of sporulation and by the finding that the absence of these enzymatic activities causes sporulation deficiency (8, 15, 18, 22, 24, 27, 28, 43, 53). Induction of the Krebs cycle at the end of the exponential growth phase presumably allows stationary-phase cells to fully metabolize by-products of glycolysis, such as pyruvate, lactate, acetate, and acetoin, yielding energy, reducing power, and biosynthetic intermediates for the synthesis of the RNA, protein, peptidoglycan, and lipid needed to form a spore (19).

As part of an assessment of the roles of individual citric acid cycle enzymes in sporulation, we have determined that a mutant defective in isocitrate dehydrogenase (ICDH) (the product of *citC* [24]), the third enzyme of the Krebs cycle, initiates sporulation but becomes blocked at stage I (28). That is, sporulating cells of the mutant assemble a ring of the cell division protein FtsZ at the sites at which polar septation would normally occur, but the septum does not form (28). This morphological block is reflected in a complementary block in gene expression. Sporulation-specific genes expressed before septation are transcribed normally in a *citC* mutant, but those genes that depend on the septation-dependent, forespore-specific, σ^F-containing form of RNA polymerase (e.g., *spoIIQ* [33]) are not (28). The progression from polar localization of the FtsZ ring to formation of the asymmetric septum is mediated in part by an unknown gene whose expression is dependent on the earliest functioning sporulation sigma factor, σ^H (32). Since σ^H is active in a *citC* mutant (28), the mechanism by which the absence of ICDH activity interferes with asymmetric septation is not known. As shown in the accompanying paper (36), further analysis of the stage I block in a *citC* mutant has revealed that inactivation of the *spoVG* gene (42, 44, 46) allows the mutant cells to overcome in part their defect in asymmetric septation and activate forespore-specific, σ^F-dependent genes. SpoVG, which is induced at the onset of stationary phase (54), acts as a transient negative regulator of the pathway leading to asymmetric septum formation (36).

Other recent studies on the sporulation deficiency of Krebs cycle mutants (8, 23, 24) have indicated that these mutants have blockages at multiple steps in the sporulation pathway. Surprisingly, a citrate synthase mutant is blocked at stage III (23), while an aconitase mutant is blocked at stage 0 (8). Since the absence of any of the first three enzymes of the Krebs cycle should cause equivalent defects in production of ATP, reducing power, and biosynthetic intermediates, these consequences of enzyme deficiency cannot explain the different phenotypes of the various mutants. To couple the developmental block in

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Ave., Boston, MA 02111-1800. Phone: (617) 636-6761. Fax: (617) 636-0337. E-mail: asonensh@opal.tufts.edu.

† Present address: Department of Biotechnology, School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka, Japan.

TABLE 1. *B. subtilis* strains used in this study

Strain	Genotype	Source, reference, or construction
JH642	*trpC2 pheA1*	J. A. Hoch
AG918	*sof-1 cat trpC2 pheA1*	A. D. Grossman
AG1431	*spo0F* Δ*S spo0B*Δ*Pst*I *trpC2*	A. D. Grossman
MO1657	Δ*amyE*::[Φ(*sspE′-lacZ*) *cat*] *trpC2 pheA1*	17
MO2051	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] *trpC2 pheA1*	33
SC432	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*]	S. Cutting
SJB219	Δ*citC*::*spc trpC2 pheA1*	28
SJB225	Φ(*spoIID′-lacZ*) *cat trpC2 pheA1*	28
SJB229	Φ(*spoIID′-lacZ*) *cat* Δ*citC*::*spc trpC2 pheA1*	28
SJB231	Δ*citZC*::*spc trpC2 pheA1*	pCS66→JH642[a]
SJB256	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] *trpC2 pheA1*	27
SJB294	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] *trpC2 pheA1*	28
SJB295	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*citC*::*spc trpC2 pheA1*	28
SJB2192	Δ*citC*::*spc trpC2 pheA1 sof-1 cat*	AG918 DNA→SJB219
SR10	Φ(*spoIID′-lacZ*) *cat*	41
AS1	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citC*::*spc sof-1 cat trpC2 pheA1*	SJB219 DNA→KMB424
KMB119	Δ*amyE*::[Φ(*spoIIQ′-lacZ*) *cat*] Δ*citZC*::*spc trpC2 pheA1*	MO2051 DNA→SJB231
KMB154	Δ*amyE*::[(P*spac-icd*$_{Sm}$[b]) *cat*] Δ*citC*::*spc trpC2 pheA1*	pKM54→SJB219
KMB165	Δ*amyE*::[Φ(*sspE′-lacZ*) *cat*] Δ*citC*::*spc trpC2 pheA1*	MO1657 DNA→SJB219
KMB170	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citC*::*spc trpC2 pheA1*	SC432 DNA→SJB219
KMB174	Φ(*spoIID′-lacZ*) Δ*citZC*::*spc trpC2 pheA1*	SR10 DNA→SJB231
KMB175	Δ*amyE*::[Φ(*sspE′-lacZ*) *cat*] Δ*citZC*::*spc trpC2 pheA1*	MO1657 DNA→SJB231
KMB176	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citZC*::*spc trpC2 pheA1*	SC432→SJB231
KMB252	*citC*::pKM112 *trpC2 pheA1*	pKM112→JH642
KMB415	*sof-1 cat trpC2 pheA1*	AG918 DNA→JH642
KMB416	*spo0A*$^+$ *cat trpC2 pheA1 cat*	AG918 DNA→JH642
KMB424	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] *sof-1 cat trpC2 pheA1*	SC432 DNA→KMB415
KMB425	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] *spo0A*$^+$ *cat trpC2 pheA1*	SC432 DNA→KMB416
KMB429	Δ*citZC::spc sof-1 cat trpC2 pheA1*	SJB231 DNA→KMB415
KMB430	Δ*citZC::spc spo0A*$^+$ *cat trpC2 pheA1*	SJB231 DNA→KMB416
KMB435	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citZC::spc sof-1 cat trpC2 pheA1*	SC432 DNA→KMB429
KMB436	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::[Φ(*cotA′-lacZ*) *cat*] Δ*citZC::spc spo0 A*$^+$ *cat trpC2 pheA1*	SC432 DNA→KMB430

[a] Plasmid pCS66 was introduced into strain JH642.
[b] The *icd*$_{Sm}$ gene encodes *S. mutans* ICDH.

a *citC* mutant with the specific metabolic and physiological changes caused by the absence of ICDH activity, we sought to identify environmental conditions and compensatory mutations that would restore sporulation competence. The results suggest that accumulation of citrate is a major contributor to the mutant phenotype. Moreover, we have found that the inhibitory effects of overabundant citrate on asymmetric septation and activation of the compartment-specific sigma factors are mainly due to lowering of extracellular pH and depletion of manganese.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains of *B. subtilis* used in this work are listed in Table 1. Use of DS medium for growth and sporulation of *B. subtilis* has been described elsewhere (24). Growth was monitored by measuring the absorbance (or optical density) at 600 nm. To quantitate spore formation, 1-ml culture samples were heated to 80°C for 10 min, diluted in sterile water, and assayed for colony formation on DS plates. *Escherichia coli* JM107 (51) was used for propagation of plasmids.

To verify the presence of the *sof-1* allele of *spo0A* in various strains, DNA from the strain in question was used to transform strain AG1431 (*spo0F spo0B*), selecting for a chloramphenicol resistance (Cam^r) marker closely linked to *spo0A*. In transformants produced by DNA from *sof-1* mutants, about 50% of the Cam^r transformants have a Spo$^+$ phenotype on DS plates.

Enzymatic assays of citrate and isocitrate. Cultures (30 ml) of wild-type or mutant cells were grown in DS medium, harvested 1 h after the entry into stationary phase (T_1), and separated by centrifugation into supernatant fluid and cell pellet fractions. The supernatant fluid was neutralized by addition of KOH. The cell pellet was washed with 20 mM Tris-HCl buffer (pH 8) containing 1 mM EDTA and then subjected to deproteinization as follows. Cells were resuspended in 4 ml of 0.3 M perchloric acid, kept on ice for 10 min, and centrifuged. A 2-ml sample of the resulting supernatant fluid was mixed with 1 ml of 0.75 M potassium carbonate and kept on ice for 15 min. After the mixture was centrifuged, the resulting supernatant fluid was used as a cell extract for enzymatic assays.

Citrate was measured by using a citric acid assay kit (Boehringer Mannheim) as described previously (8). For measurement of isocitrate, a 50-μl sample was added to 950 μl of ICDH assay buffer (50 mM Tris-HCl [pH 7.5], 1 mM $MnCl_2$, 1 mM $NADP^+$). After purified *B. subtilis* ICDH (0.014 unit) (35) was added to the reaction mixture, NADPH production, coupled to conversion of isocitrate to 2-ketoglutarate, was monitored at 340 nm. The concentration of isocitrate was calculated from a standard curve based on commercially prepared isocitrate. One unit of enzymatic activity was defined as the amount of enzyme that produces 1 μmol of NADPH per min with a saturating concentration (0.2 mM) of DL-isocitrate.

Measurements of intracellular metabolite pools by HPLC analysis. For each time point, a 5-ml sample of a culture in DS medium was removed and the cells were collected rapidly by filtration through a Millipore HA filter (pore size, 0.45 μm) that had previously been boiled for 10 min in 1 liter of water to remove contaminants. The filter was washed with ice-cold ultrapure water and plunged into 5 ml of boiling ultrapure water. When the volume was reduced to about 0.5 ml, the liquid was cooled, transferred to a microcentrifuge tube, and stored at −80°C. For analysis, each sample was thawed, centrifuged to remove debris, and passed through a 0.22-μm-pore-size high-performance liquid chromatography (HPLC) filter. The HPLC analyses (4) were performed on a Dionex DX-500-Microbore system equipped with the Pulsed Electrochemical Detector (ED40), which was operated in conductivity mode with an Anion Self-Regenerating Suppressor (ASRS-1) and the variable wavelength detector (AD20) set at 260 nm. The Dionex IonPac AS11 column (2 by 250 mm), equipped with a guard column, was employed with the following sodium hydroxide gradient elution profile. The column was first equilibrated for 10 min with 2 mM NaOH. Following sample injection, the NaOH gradient was increased linearly from 2 to 14 mM from 11 to 20 min, then increased with a slightly concave curve from 14 to 28 mM NaOH from 20 to 28 min, increased linearly from 28 to 80 mM NaOH from 28 to 35 min, and finally was held at 80 mM NaOH from 35 to 40 min. The flow rate was set at 0.5 ml/min, and a 10-μl sample injection loop was used. Data were analyzed by using PeakNet, release 4.11A, chromatography software program

(Dionex Corp.). In an independent run, each sample was mixed with a set of chromatography standards and analyzed.

To calculate intracellular metabolite concentrations, a standard curve that relates cell number to optical density at 600 nm (OD_{600}) was prepared and an internal volume of 1.1×10^{-9} μl per cell was assumed (50).

Deletion-insertion mutation of *citZC*. Plasmid pCS66 (23) contains the first 30 amino acids of the *citZ* gene and the last 130 amino acids of the *citC* gene, with the spectinomycin cassette (*spc*) inserted in-between. The orientation of the *spc* gene in pCS66 is such that transcription is in the same direction as that of the *citZ* and *citC* genes. S. Jin introduced linearized pCS66 by transformation into *B. subtilis* JH642 to create strain SJB231. Transformants that were spectinomycin resistant and chloramphenicol sensitive were shown by Southern blot analysis to have undergone double-crossover recombination.

Expression of the *Streptococcus mutants icd* gene in *B. subtilis*. The *S. mutans icd* gene, encoding ICDH, was amplified by PCR, using plasmid pJG400 (9) as the template and oligonucleotide primers that allowed introduction of a *B. subtilis* consensus ribosome binding site (RBS) just upstream of the *icd* coding sequence. The PCR product (1.2 kb) was inserted at the modified *Eco*RV site of pT7Blue(R) (Novagen, Inc.) (generating pKM35), and then subcloned between the *Sal*I and *Sac*I sites of pBluescript SK(−) (pSK−) (Stratagene, Inc.), to create pKM42. To eliminate possible PCR-derived errors, we replaced the 3′ end of the PCR-amplified *icd* gene (a *Hin*dIII-*Sac*I fragment of about 1.2 kb) with the corresponding region of the original *icd* gene from pKM34 (see below), to create pKM49. The *Sal*I (blunt-ended with the Klenow enzyme)-*Sac*I fragment of pKM49, which contains the *S. mutans icd* gene preceded by the *B. subtilis* RBS, was inserted between the *Hin*dIII (blunt-ended) and *Sac*I sites of pAF3 (16), creating pKM54. After transformation of *B. subtilis* Δ*citC* mutant cells with pKM54 and selection for chloramphenicol resistance, we isolated clones in which *icd* was integrated at the *amyE* locus and its expression was under the control of the *spac* promoter (52).

pKM34 was constructed by ligating a 3.5-kb *Hin*dIII-*Eco*RI fragment (*′acn citZ icd*) of pJG400 to pSK− which had been cut with the same enzymes.

Cloning of the *citC* gene. Plasmid pKM3 (a pSK- derivative) has a *Hin*dIII-*Eco*RI fragment (2.2 kb) of pCS60 (24), which contains the 3′ end of the *citC* gene (about 0.7 kb), the *citH* gene, and the 5′ end of the *phoP* gene. The entire *citC* gene was cloned by joining the *Sac*II (blunt-ended)-*Hin*dIII fragment (0.7 kb) of pCS71 (24) to pKM3 which had been cut with *Sal*I (blunt ended) and *Hin*dIII, resulting in pKM4. Plasmid pKM11 was constructed by inserting a 1.4-kb *Dra*I-*Ear*I fragment, which contains the entire *citC* gene without its gene-specific promoter, into the *Sma*I site of pBB544, a *B. subtilis* integrative vector (2). The orientation of the *citC* gene in pKM11 was the same as that of the *lacZ* gene in pBB544.

Site-directed mutagenesis. An essential serine (residue 103) in the active site of *B. subtilis* ICDH (35) was changed to aspartic acid by PCR-mediated mutagenesis (20) with pKM11 as the template and the following oligonucleotide primers: primer a, 5′-GACACCTGTCGGCGGCGGTATCCGTGATTTGAACGTAGCGCTC; primer b, 5′-GAGCGCTACGTTCAAATCACGGATACCGCCGCCGACAGGTGT; primer c, 5′-GGAAACAGCTATGACCATG (vector-derived sequence, upstream of *citC*); and primer d, 5′-GTAAAACGACGGCCAGT (vector-derived sequence, downstream of *citC*). The mutation is underlined in the complementary mutagenic primers a and b. In brief, initial PCRs using oligonucleotide primers a and d or b and c produced fragments with overlapping ends. The entire *citC* gene containing the mutation was generated in a subsequent PCR using oligonucleotides primers c and d. In all PCR reactions, the Expand High Fidelity PCR system (Boehringer Mannheim) was used. After the amplified mutant *citC* gene was digested with *Bam*HI and *Eco*RI, the DNA fragment was cloned between the *Bam*HI and *Eco*RI sites of pBB544 (Neo^r) (2), creating pKM94. To remove other possible mutations before direct DNA sequencing, a 0.7-kb *Hin*dIII-*Cla*I (blunt-ended) fragment of pKM94 (3′ end of *citC*, downstream of the active-site mutation) was replaced with a *Hin*dIII-*Eco*RV fragment of pKM11, resulting in pKM112.

Plasmid pKM69, a derivative of the *amyE* integration vector pAF3 (16) was constructed as follows. The region of the P*spac*A(1)G-*E. coli icd* gene on the chromosome of strain KMB15 (28) was amplified by PCR, cloned in the modified *Eco*RV site of pT7Blue(R), and after cleavage with *Eco*RI and *Sac*I, ligated to pAF3 that had been cleaved with the same enzymes, creating pKM67. The PCR-amplified *E. coli icd* gene preceded by the *B. subtilis* consensus RBS (*Hin*dIII-*Sac*I fragment) in pKM67 was replaced with the modified *icd* gene (*Hin*dIII-*Sac*I fragment) cloned in pKM12B (28), to create pKM69.

To test the function of *B. subtilis* ICDH(S103D), we constructed a plasmid (pKM118) having the mutated *citC* gene fused to the *spacA*(1)G promoter (28) in pKM69 and integrated this plasmid at the *amyE* locus in a Δ*citC* mutant (SJB219). The resulting strain was a glutamate auxotroph, indicating that the point mutation disrupts ICDH enzymatic activity. Next, strain JH642 was transformed with pKM112. After selection for Neo^r and screening for Glt^- clones, a single-crossover mutant (KMB252) was obtained for further study.

Protein analysis. Cells were harvested from an 80-ml culture, washed with 20 mM Tris-HCl buffer (pH 7.4) containing 1 mM citrate, 5 mM $MnCl_2$, 5 mM β-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol, and resuspended in 2 ml of the same buffer. Cells were disrupted by sonication and centrifuged to remove cell debris. The supernatant fluid was used as a crude extract in this study. Crude extract (3 μg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then electrotransferred to Immobilon-P membranes (Millipore Corp.) with a Mini Trans-Blot transfer cell (Bio-Rad Laboratories) according to the supplier's instructions. ICDH was detected by exposure to rabbit antibodies against *B. subtilis* ICDH (37) and subsequent alkaline phosphatase staining (26).

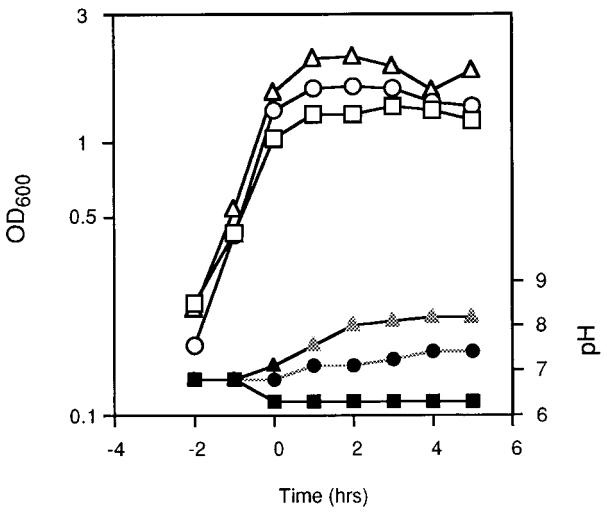

FIG. 1. Growth and pH in DS medium. Strains JH642 (*cit*$^+$; triangles), SJB219 (Δ*citC*; squares), and SJB231 (Δ*citZC*; circles) were grown in DS medium, and samples were removed at the indicated times for measurement of OD_{600} and, after removal of cells by centrifugation, for determination of the pH of the medium. The time of onset of stationary phase is designated as T_0.

Other methods. DNA manipulations were performed as described previously (24, 36). Transformation of cells of *E. coli* and *B. subtilis* and β-galactosidase assays were performed as described previously (24). Thin-section electron microscopic analysis was performed as described previously (28), with the assistance of A. Brown-Cormier, Electron Microscopy Unit, Department of Anatomy and Cellular Biology, Tufts University. All oligonucleotides used in this study were synthesized by M. Berne, Tufts University Protein and Nucleic Acid Analysis Facility.

RESULTS

Growth and sporulation of a *citC* mutant. In nutrient broth sporulation (DS) medium, strain SJB219 (Δ*citC*::*spc*) grew at about the same rate as did wild-type cells (Fig. 1) but reached stationary phase at a lower cell density ($\sim 1 \times 10^8$ per ml versus $\sim 5 \times 10^8$ per ml). Moreover, while wild-type cultures lost 20 to 40% of their cells to lysis in stationary phase, the *citC* mutant cultures lost 60 to 90% of their cells. As a result of these effects, the titer of viable *citC* mutant cells at T_{20} was ≤10% of the wild-type titer. Of the surviving cells, only 0.1 to 1% formed heat-resistant spores and total spore yield in the mutant was 0.01 to 0.1% of that in the isogenic wild-type strain (Table 2). As shown previously, the specific stage of blockage of sporulation in the *citC* mutant is stage I, that is, the step during which the asymmetric septum forms (28). As a result, the *citC* mutant does not express genes whose transcription is dependent on σ^F (e.g., *spoIIQ*) or any sporulation genes expressed later (28). Despite the fact that the Δ*citC* mutant does not grow to as high a cell density as the wild type does, it is able to initiate sporulation, as shown by the normal levels of expression of Spo0A-phosphate-dependent genes and bipolar localization of FtsZ rings seen in the mutant (28).

During growth of the wild-type cells in DS medium, the pH of the culture medium dropped transiently from 7.1 to about 6.8 and then rose during stationary phase to 8.0 to 8.5 (Fig. 1). In the *citC* mutant culture, however, the pH dropped to 6.3 to 6.5 and was not reversed (Fig. 1). This result would be consistent with the inability of these cells to import and oxidize acidic

TABLE 2. Sporulation of tricarboxylic acid cycle mutants

Addition to medium[a]	Genotype	Maximum OD_{600}[b]	Maximum titer[c]	Titer at T_{20}[d]	Spore titer at T_{20}[d]	Sporulation efficiency[e]	Sporulated fraction[f]	Relative sporulation frequency[g]
None	*cit*$^+$	1.9	8.5×10^8	4.7×10^8	4.6×10^8	0.54	0.98	(1.0)
	Δ*citC*	1.2	1.4×10^8	1.4×10^7	5.7×10^4	0.0004	0.004	0.0001
	Δ*citZC*	1.5	4.7×10^8	7.7×10^7	1.3×10^7	0.028	0.17	0.03
	Δ*citZC sof-1*	1.5		1.4×10^8	1.1×10^8		0.78	0.24
$MnCl_2$	*cit*$^+$	2.0	3.5×10^8	3.3×10^8	3.0×10^8	0.85	0.91	(1.0)
	Δ*citC*	1.1	1.6×10^8	5.3×10^7	1.5×10^6	0.009	0.028	0.005
HEPES	*cit*$^+$	1.6	5.8×10^8	6.9×10^8	6.4×10^8	1.10	0.93	(1.0)
	Δ*citC*	1.1	3.1×10^8	4.4×10^7	3.3×10^6	0.001	0.075	0.005
Glt	*cit*$^+$	2.3		5.4×10^8	5.2×10^8		0.96	(1.0)
	Δ*citC*	1.6		7.7×10^7	7.2×10^6		0.09	0.012
Glucose	*citC*$^+$ *sof-1*	2.1	7.2×10^8	8.2×10^8	8.2×10^8	1.13	1.00	(1.0)
	Δ*citC sof-1*	2.2	2.1×10^8	7.0×10^6	1.2×10^5	0.006	0.017	0.0001

[a] Final concentrations of additions to DS medium were as follows: 0.75 mM $MnCl_2$, 50 mM HEPES (pH 8), 0.2% glutamate (Glt), and 0.1% glucose.
[b] Maximum OD_{600} was defined as the highest OD_{600} in early stationary phase.
[c] Maximum titer was the concentration of viable cells at the highest cell density.
[d] At T_{20}, viable-cell titer was determined and samples of cultures were heated at 80°C for 10 min to test for heat-resistant spore formation.
[e] Sporulation efficiency is defined as the ratio of heat-resistant CFU at T_{20} to maximum titer in early stationary phase.
[f] Sporulated fraction is defined as the ratio of heat-resistant CFU to total viable cell titer at T_{20}.
[g] Relative sporulation frequency is defined as heat-resistant spore titer in *cit* mutant cultures relative to that in *cit*$^+$ cultures.

metabolites of pyruvate generated and excreted during exponential growth phase (17a). In DS medium, the primary carbon and energy sources are amino acids and glycerol (17b).

Effect of a *citC* missense mutation. To know whether the requirement for the *citC* gene during sporulation is a reflection of the enzymatic activity of ICDH or some other role of the protein, we created a mutant strain having a single amino acid alteration (S103D) in the active-site serine (see Materials and Methods). Immunoblotting analysis indicated that ICDH (S103D) is stable in *B. subtilis* (data not shown). The mutant strain was totally devoid of ICDH activity, formed spores at 0.01% of the frequency of the wild-type strain, and failed to express a *spoIIQ'-lacZ* fusion, as is the case for the Δ*citC* mutant, implying that the enzymatic activity of *B. subtilis* ICDH is the only function necessary for sporulation.

In previous work, we showed that the *E. coli* gene for ICDH could fully compensate for the absence of the *B. subtilis citC* gene with respect to growth and sporulation (28). To extend this analysis, we expressed in a *B. subtilis* Δ*citC* mutant the *icd* gene from *S. mutans* (9). Again, the foreign gene for ICDH was fully capable of compensating for the absence of *B. subtilis* ICDH (data not shown). Taken together, these results support the idea that it is the enzymatic activity of ICDH, rather than some other hypothetical function of the protein, that is critical for sporulation.

Metabolic defects in a *citC* mutant. In addition to producing 2-ketoglutarate, *B. subtilis* ICDH has the specific function of generating NADPH, a substrate for synthesis of fatty acids and some amino acids. To see whether this role of ICDH is important in sporulation, we supplemented the medium with the branched-chain, unsaturated fatty acids 13-methylmyristic acid and 12-methyltetradecanoic acid (each at 16 μg per ml) and a mixture of cysteine, arginine, lysine, methionine, and isoleucine (each at a concentration of 50 μg per ml). No increase in cell survival or sporulation was seen. As noted above, the *S. mutans* ICDH fully compensates for the absence of *B. subtilis* ICDH. Since *S. mutans* ICDH produces NADH rather than NADPH (9), the NADPH-producing function of ICDH does not appear to be essential for growth or sporulation in DS broth.

As a component of the Krebs cycle, ICDH participates in production of ATP and in gluconeogenesis. To assess the extent to which these roles of ICDH explain the stage I block of a *citC* mutant, we first measured ATP levels in mutant and wild-type cells (Table 3). At T_0, the time of transition from exponential growth to stationary phase, the ATP level in both mutant and wild-type cells was approximately 1.5 mM (Table 3). One hour later, at T_1, when the asymmetric septum is formed, the ATP concentration was higher in mutant cells than in wild-type cells (Table 3). Next, we added glucose to mutant and wild-type cells to provide an alternative source of ATP and to obviate the need for gluconeogenesis. Because glucose is normally a potent inhibitor of sporulation, we used a strain carrying a *spo0A* mutation, *sof-1*, that prevents inhibition of sporulation by glucose (21). Addition of glucose to *citC*$^+$ *sof-1* and Δ*citC sof-1* cultures allowed growth of the Δ*citC* mutant to a higher cell density but caused only a very small increase in sporulation (Table 2) or *spoIIQ'-lacZ* expression (data not shown) by the *citC* mutant strain. We conclude that the block at stage I seen in *citC* mutant cells is not solely caused by a deficiency in ATP or gluconeogenesis or both.

TABLE 3. Metabolite pools in Δ*citC* mutant and wild-type cells determined by HPLC analysis[a]

Strain (relevant genotype)	Time	AMP concn (mM)	ADP concn (mM)	ATP concn (mM)	Phosphoenolpyruvate concn (mM)
JH642 (*cit*$^+$)	T_0	4.8	4.9	1.5	0.94
	T_1	3.1	3.5	3.0	1.3
SJB219 (Δ*citC*)	T_0	7.9	1.5	1.5	1.5
	T_1	4.8	6.3	3.9	2.3

[a] HPLC analysis of metabolite pools was performed as described in Materials and Methods.

TABLE 4. Measurements of citrate and isocitrate[a]

Strain	Intracellular concn by:				Extracellular concn by enzyme assay	
	Enzyme assay		HPLC analysis			
	Citrate (μmol/OD_{600})[b]	Isocitrate (μmol/OD_{600})[b]	Citrate (mM)	Isocitrate (mM)	Citrate (mM)	Isocitrate (mM)
cit$^+$	<0.073	<0.024	1.3	0.17	0.062	<0.02
Δ*citC*	0.86	<0.042	22	3.6	0.89	0.29
Δ*citZC*	0.45	<0.029			0.12	<0.02

[a] Cells were grown to stationary phase (T_1) in DS medium, and samples for measurements of citrate and isocitrate were prepared by the methods described in Materials and Methods.

[b] Total citrate or isocitrate (in micromoles) in cell extracts prepared from 30-ml cultures was normalized by dividing by the OD_{600} of the culture at the time of harvesting.

Metabolite accumulation. A defect in any metabolic pathway can be predicted to cause not only a deficiency in the products of the pathway but also accumulation to abnormal levels of one or more intermediates upstream of the interrupted step. We therefore examined the concentrations of citrate and isocitrate in *citC* mutant cells. The measurement of these metabolites by enzymatic assays (see Materials and Methods) showed that the culture medium of the *citC* mutant cells at T_1 contained both citrate and isocitrate at 10- to 15-fold-higher levels than those found in the wild-type culture medium (Table 4). The intracellular concentration of citrate was also 10-fold higher in the *citC* mutant than in a wild-type extract, but the intracellular concentration of isocitrate was too low to be measured accurately by this assay (Table 4). Therefore, we conducted a more-detailed analysis of intracellular metabolites by HPLC (see Materials and Methods). The HPLC analysis indicated that the *citC* mutant had 15- to 20-fold-higher intracellular concentrations of citrate and isocitrate than did wild-type cells (Table 4). This result fits with the absence of ICDH activity and the fact that the equilibrium of aconitase greatly favors conversion of isocitrate to citrate. It is noteworthy that all other pools examined by HPLC, including ATP, ADP, AMP, and phosphoenolpyruvate were at least as high in the *citC* mutant as in wild-type cells (Table 3). We do not know whether variations in the ratios of the nucleotides in any given extract are significant.

Phenotype of a *citZ citC* mutant. To see whether the excessive accumulation of citrate and isocitrate is responsible for the lower growth yield and sporulation deficiency of the *citC* mutant, we constructed a mutant strain from which the major citrate synthase gene (*citZ*) and the *citC* gene were deleted simultaneously. Concentrations of citrate and isocitrate in the culture medium were reduced to near-wild-type levels (Table 4). Note that this strain continues to produce citrate at a low rate due to the activity of the *citA* gene product, a second isozyme of citrate synthase (24), but fails to convert citrate or isocitrate to 2-ketoglutarate because of the *citC* mutation. This Δ*citZC* strain (SJB231) grew to a higher cell density than did the *citC* mutant and survived better in stationary phase (final titer of 7.7×10^7 per ml [Table 2]), supporting the idea that accumulation of citrate is in some way harmful to cell survival in stationary phase. One of the consequences of accumulation of citrate might be to lower the pH of the culture medium. In fact, the pH of the culture medium of the *citZC* mutant reached about 7.5 (about 1 unit higher than that of the *citC* mutant but still 1 unit lower than in the wild-type strain) in stationary phase (Fig. 1). The double-mutant cells produced 100 times more spores per ml than did the *citC* mutant cells (Table 2), but the majority of the double-mutant cells were still blocked at stage I of the developmental pathway as determined by measurements of *spoIIQ′-lacZ* expression (see below).

The finding that substantial reduction in citrate synthesis partially suppresses the defect in growth and sporulation of a *citC* mutant was confirmed by the isolation of spontaneous variants of SJB219. Colonies of SJB219 on DS plates are translucent due to substantial lysis of the cells. After several days of incubation, less-translucent papillae arose on many of the colonies. One such spontaneous variant was analyzed in detail. The mutation responsible for altered colony morphology was approximately 90% linked to Δ*citC*::*spc* by transformation, suggesting that it was likely to lie within the *citZCH* operon. The double mutant grew and formed spores at the same frequency as SJB231 (Δ*citZC*) (Table 2). Extracts of the variant strain showed that it had the same level of malate dehydrogenase (CitH) activity as in SJB219 but essentially no citrate synthase (CitZ) activity. Thus, the mechanism of suppression is apparently a reduction in accumulation of citrate due to inactivation of *citZ*. A mutant strain with deletions of both citrate synthase genes (*citA* and *citZ*) and of *citC* had a phenotype very similar to that of the *citZC* deletion strain (data not shown). Reduction of citrate synthase activity also serves to cure in part the growth defect of an ICDH mutant of *E. coli* (31).

Effects of divalent cations. Accumulation of citrate in the *citC* mutant might also interfere with sporulation by chelating divalent cations and inhibiting the activity of one or more metal ion-dependent enzymes. We therefore added excess $MnCl_2$, $MgSO_4$, and $FeSO_4$, separately or together, to DS medium. Raising the $MnCl_2$ concentration in the medium from 0.01 mM (the standard concentration) to 0.5 or 0.75 mM had no significant effect on wild-type cells but increased sporulation of the *citC* mutant by 100- to 1,000-fold (Table 2). Higher concentrations inhibited sporulation. Similarly, increasing the $FeSO_4$ concentration from 0.001 to 0.05 mM increased spore formation by the *citC* mutant by 50- to 100-fold. Supplementation of $MnCl_2$ and $FeSO_4$ simultaneously had no additive effect. Addition of supplementary $MgSO_4$ did not increase sporulation efficiency. Thus, sporulation of the *citC* mutant seems to be limited in part by availability of certain divalent cations, presumably because of excess citrate accumulation.

TABLE 5. 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
	HEPES	<10	<10	<10	<10
	$MnCl_2$	25	22	<10	<10
	HEPES + $MnCl_2$	20	13	<10	<10
Δ*citC sof-1*	None	<10			<10
Δ*citZC*	None	26	13	<10	<10
Δ*citZC 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 β-galactosidase activities (in Miller units) for the wild-type strains were as follows: *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.

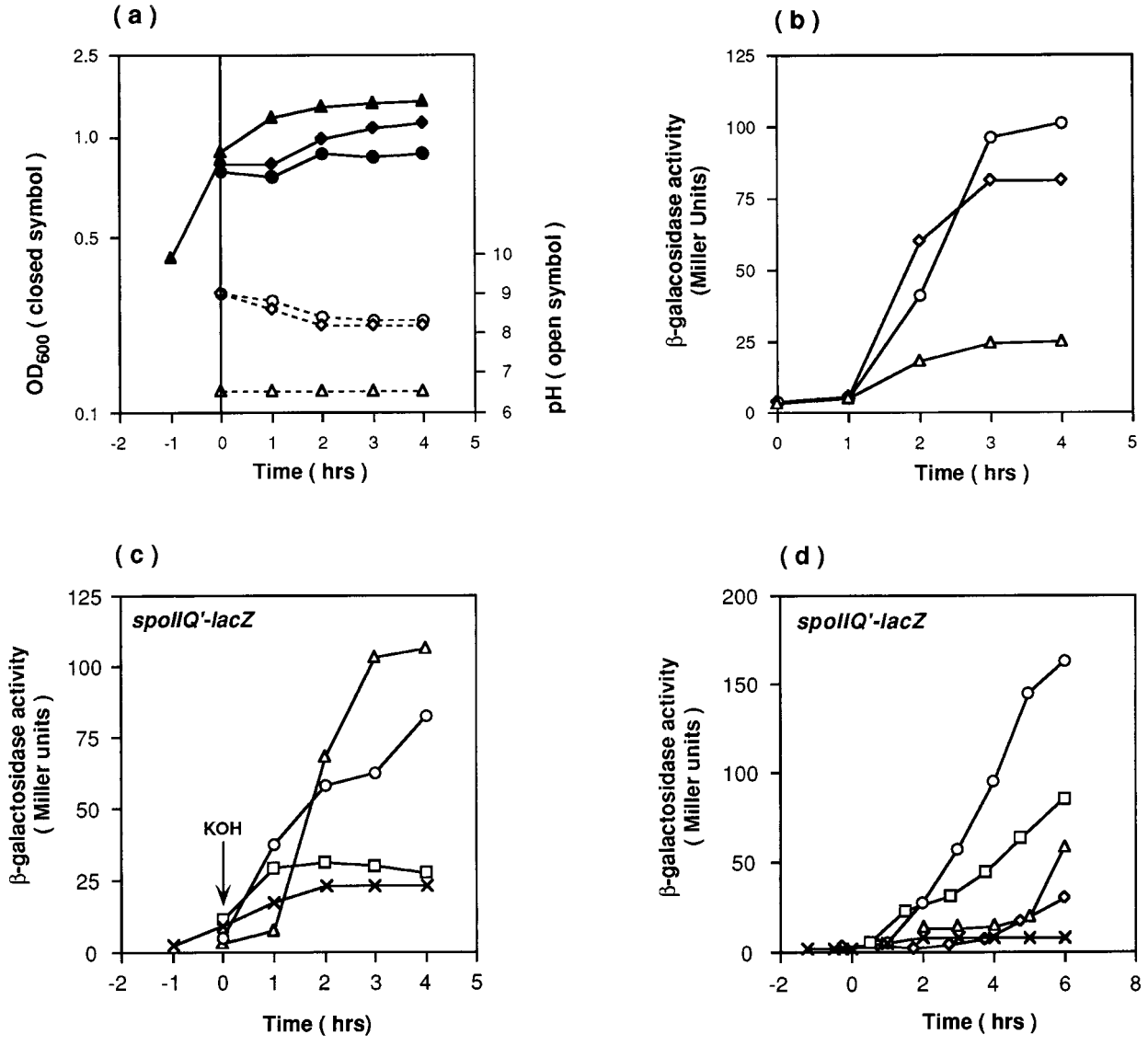

FIG. 2. Effect of KOH on *spoIIQ'-lacZ* expression in a Δ*citC* mutant. (a and b) Cells of strain SJB295 (Δ*citC*) were grown in DS broth (triangles). After 5 mM KOH (circles) or NaOH (diamonds) was added to the culture at T_0, the OD_{600} (panel a, closed symbols), pH of the medium (panel a, open symbols) and *spoIIQ'-lacZ* expression (panel b) were measured. (c) Different concentrations of KOH were added to the culture at T_0 (×, no addition; □, 2.5 mM; ○, 5 mM; △, 6.25 mM). (d) KOH (5 mM) was added to the culture at $T_{-1.25}$ (◇), $T_{-0.5}$ (□), T_0 (○), or T_1 (△). ×, control (no KOH added).

While supplementation with divalent cations did not restore expression of *spoIIQ'-lacZ* to the majority of the cells, it did allow a detectable increase in expression of both *spoIIQ* and *spoIID* (Table 5).

Effect of pH alteration. While searching for physiological conditions that would restore *spoIIQ'-lacZ* expression to a *citC* mutant, we unexpectedly found that addition of KOH to unbuffered DS medium has a significant stimulatory effect on *spoIIQ* expression. As shown in Fig. 2b, when KOH was added to the *citC* mutant culture to give a final concentration of 5 mM, high-level *spoIIQ* expression was observed, despite the fact that the growth rate dropped slightly over the same period. The pH of the culture medium just after KOH addition was almost 9 and then decreased to 8.0 to 8.5 over 4 h (Fig. 2a). The optimum concentration of KOH was determined to be 5 to 6.25 mM (Fig. 2c); a higher concentration of KOH (for example, 7.5 mM) caused aggregation of the cells, and no *spoIIQ* expression was observed. Altering the time of addition of KOH showed that KOH had its strongest activating effect on *spoIIQ'-lacZ* expression when it was added to the mutant culture at around T_0 (Fig. 2d), while such addition caused delayed expression of *spoIIQ'-lacZ* in the wild-type strain (data not shown). The restoration of *spoIIQ'-lacZ* expression in the *citC* mutant is apparently due to raising the pH rather than increasing availability of K^+ ions, since NaOH had the same effect as KOH (Fig. 2b) and KCl had no stimulatory effect (data not shown). These results suggest that raising the pH at T_0 allows the *citC* mutant to bypass the stage I block in sporulation.

Under these conditions, however, the sporulation frequency of the *citC* mutant was not improved. Electron microscopy showed that about 50% of the cells were able to pass the stage I block in sporulation and that about half of these cells reached the engulfed forespore stage by T_6 (Fig. 3). Mother cell-specific, σ^E-dependent *spoIID* expression (41) and forespore-specific, σ^G-dependent *sspE* expression (12) were partially acti-

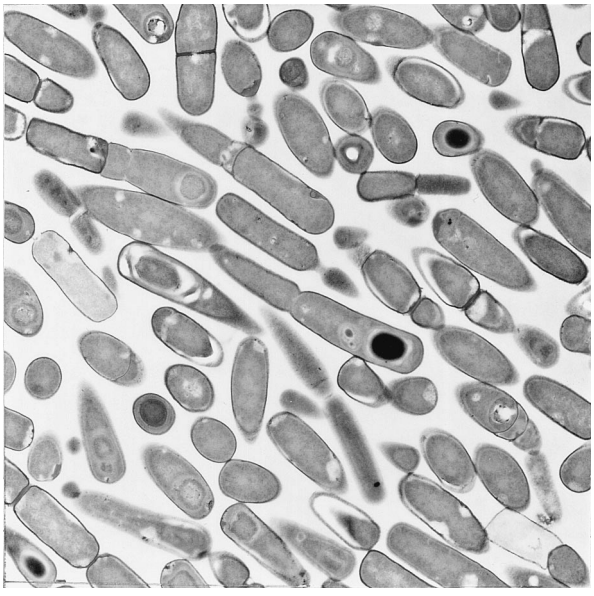

FIG. 3. Thin-section electron microscopic analysis. Cells of a Δ*citC* mutant strain (SJB219) were grown in DS medium to the end of exponential growth phase (T_0), and 5 mM KOH was added to the culture. KOH-treated cells were harvested 6 h after the addition of KOH, and samples were prepared for transmission electron microscopy.

vated by KOH addition, but σ^K-dependent expression of the *cotA* gene (45) was still at a very low level (data not shown).

To assess further the importance of extracellular pH control in growth and sporulation of the *citC* mutant, we added HEPES (50 to 100 mM) or Tris (50 to 100 mM) buffers to DS medium to maintain a constant pH of 8.0 to 8.5. In such media, neither growth nor survival of the *citC* mutant was significantly improved, but the fraction of surviving cells that formed spores increased substantially (Table 2). Expression of *spoIIQ′-lacZ* was not recovered, however (Table 5), even when the buffer was added at T_0 (data not shown). Simultaneous addition of HEPES or Tris (50 mM) and excess $MnCl_2$ (0.75 mM) had no additive effect on heat-resistant spore formation (data not shown) or on *spoIIQ′-lacZ* expression (Table 5).

Effects of supplementation of metabolites. The failure of *citC* mutant cells to grow to high titer is probably due primarily to the failure to utilize fully the by-products of pyruvate metabolism (acetate, lactate, and citrate). The effect might also be due in part to the failure to synthesize adequate 2-ketoglutarate. If so, addition to the medium of metabolites produced from 2-ketoglutarate might alleviate the defect. When the *citC* mutant cells were grown in DS medium supplemented with 0.2% glutamate, they grew to a higher density (as did the wild-type cells) and, as a result, achieved a high titer in late stationary phase. Moreover, 9% of the surviving cells were heat-resistant spores (≤2% of the wild-type spore production) (Table 2). Two types of explanations for this result can be imagined. On the one hand, *citC* mutants in DS medium might be limited for glutamate or 2-ketoglutarate. This seems very unlikely, however, since nutrient broth, the only organic constituent of DS medium, is made from boiled meat and contains substantial quantities of most amino acids, including glutamate. In fact, DS medium contains sufficient glutamate to permit normal growth and sporulation of a glutamate synthase mutant, which is an absolute glutamate auxotroph (3). In addition, glutamate dehydrogenase activity is induced in DS medium (3), allowing conversion of glutamate to 2-ketoglutarate. On the other hand, the addition of glutamate might have indirect effects on cell physiology. In fact, glutamate addition caused the pH of the culture medium to rise during growth so that even in *citC* mutant cells, it was >8.0 in stationary phase. Moreover, the effect of glutamate was mimicked by and additive with supplementation with succinate or aspartate (data not shown). Although addition of each of these compounds may help the *citC* mutant to sporulate for a different reason, the simplest interpretation is that they all work by causing extracellular pH to rise.

Synergistic effects of mutations in *citZ* and *spo0A*. A mutant strain with both *citZ* and *citC* deleted was able to pass through stage I of sporulation and express, to a significant extent, genes dependent on σ^F and, to a lesser extent, σ^E (Fig. 4 and Table 5). The double mutant did not express genes dependent on σ^G or σ^K, however (Fig. 4 and Table 5). In an attempt to find conditions that would allow this double-mutant strain to progress further in the sporulation pathway, we tested the effects of introducing additional mutations. Surprisingly, complete restoration of σ^K-dependent *cotA′-lacZ* expression and nearly complete restoration of spore formation were seen when the *sof-1* allele of the *spo0A* gene was introduced into the Δ*citZC* strain (Fig. 4C and Tables 2 and 5). This was the only combination of conditions or mutations that had such a strongly suppressive effect on the absence of ICDH. As shown above, the *sof-1* mutation had no suppressive effect on a Δ*citC* single mutant strain; its ability to suppress seems dependent on a reduction in citrate synthesis and accumulation.

DISCUSSION

Abnormal accumulation of citrate and isocitrate seems to be responsible for the earliest sporulation defect, i.e., lack of asymmetric septation, in a *citC* mutant. Citrate and isocitrate accumulate to unusually high levels because they cannot be further metabolized in the absence of ICDH. No citrate lyase or isocitrate lyase or other citrate- or isocitrate-degrading activity has been found in *B. subtilis* (13, 37), and examination of the *B. subtilis* genome sequence (30) revealed no homolog of *E. coli* citrate lyase, isocitrate lyase, or malate synthase. Such accumulation of citrate and isocitrate apparently has two negative effects on sporulation. First, the pH of the medium remains low (at 6.5) as cells enter stationary phase. By contrast, the pH of the culture medium of wild-type cells rises in stationary phase to 8.5 due, at least in part, to uptake of previously excreted organic acids, their conversion to citrate, and their subsequent oxidation. Asymmetric septation occurs at a time (approximately T_1) when the extracellular pH is 7.5 to 8.0. In the *citC* mutant, extracellular organic acids, such as acetate and lactate, can presumably be taken up and converted to citrate and isocitrate, but no further metabolism ensues and citrate and isocitrate are excreted, maintaining low environmental pH. We have not measured the effect of the absence of ICDH on intracellular pH. In wild-type cells, internal pH is kept almost constant during growth and during most of sporulation (6, 7, 29, 34).

Raising external pH restored sporulation gene expression to a significant fraction of the *citC* mutant cells only when it was accomplished by the addition of inorganic alkali or certain Krebs cycle-derived compounds (glutamate, succinate, and aspartate); organic buffers affected only a small minority of the cell population even though they raised the extracellular pH to 8.0 or higher. We do not know either the basis for this difference or the mechanism that seems to link extracellular pH and asymmetric septation. It is possible that efficient uptake of the pH-altering agent is critical.

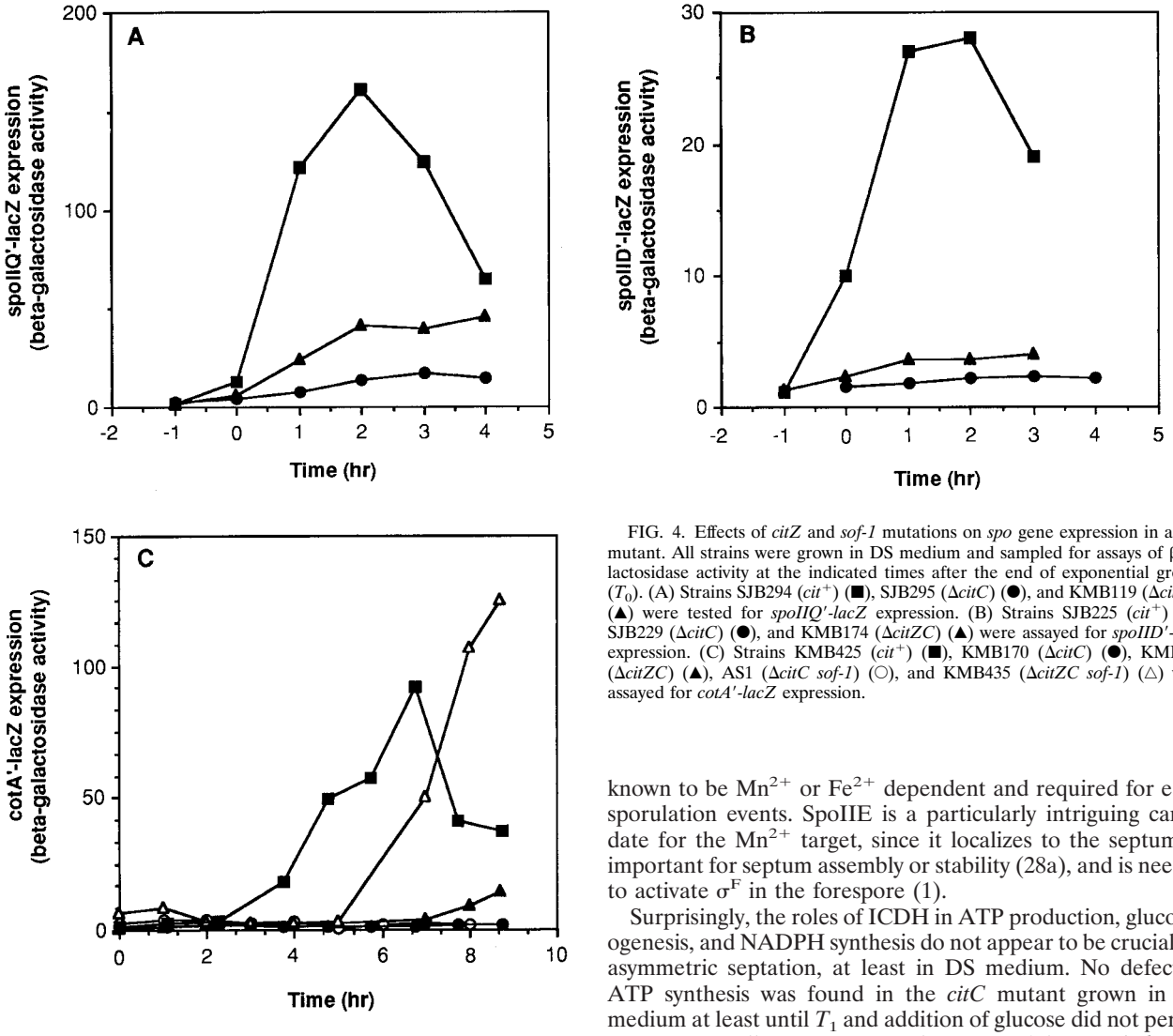

FIG. 4. Effects of *citZ* and *sof-1* mutations on *spo* gene expression in a *citC* mutant. All strains were grown in DS medium and sampled for assays of β-galactosidase activity at the indicated times after the end of exponential growth (T_0). (A) Strains SJB294 (*cit*$^+$) (■), SJB295 (Δ*citC*) (●), and KMB119 (Δ*citZC*) (▲) were tested for *spoIIQ'-lacZ* expression. (B) Strains SJB225 (*cit*$^+$) (■), SJB229 (Δ*citC*) (●), and KMB174 (Δ*citZC*) (▲) were assayed for *spoIID'-lacZ* expression. (C) Strains KMB425 (*cit*$^+$) (■), KMB170 (Δ*citC*) (●), KMB436 (Δ*citZC*) (▲), AS1 (Δ*citC sof-1*) (○), and KMB435 (Δ*citZC sof-1*) (△) were assayed for *cotA'-lacZ* expression.

An effect of low external pH on sporulation gene expression has also been seen by others (6, 7). In DS medium supplemented with glucose and glutamine, the pH of the medium drops to 5.5, at which point the accumulation of σ^H and expression of σ^H-dependent genes is inhibited. The same mechanism is unlikely to be responsible for the phenotype of a *citC* mutant, since the pH does not reach 5.5 and expression of σ^H-dependent genes is not inhibited (28).

The second effect of excess citrate and isocitrate appears to be chelation of Mn^{2+} (and perhaps Fe^{2+}). Since the block in asymmetric septation can be bypassed in part of the cell population by addition of KOH or NaOH, we suggest that low pH is the primary deterrent to asymmetric septation in *citC* mutant cells. However, Mn^{2+} must also contribute to asymmetric septation, since expression of septation-dependent genes in a *citC spoVG* double mutant is raised significantly by supplementation with excess Mn^{2+} even though the pH remains low (36). The mechanistic basis of the cation effect is unknown. At least five enzymes of *B. subtilis*, aconitase (14), fructose-1,6-diphosphate aldolase (14), ICDH (37, 40), phosphoglycerate phosphomutase (38), and SpoIIE, a phosphatase that activates σ^F by helping to liberate it from its inhibitor protein (10), are known to be Mn^{2+} or Fe^{2+} dependent and required for early sporulation events. SpoIIE is a particularly intriguing candidate for the Mn^{2+} target, since it localizes to the septum, is important for septum assembly or stability (28a), and is needed to activate σ^F in the forespore (1).

Surprisingly, the roles of ICDH in ATP production, gluconeogenesis, and NADPH synthesis do not appear to be crucial for asymmetric septation, at least in DS medium. No defect in ATP synthesis was found in the *citC* mutant grown in DS medium at least until T_1 and addition of glucose did not permit *citC* mutant cells to septate. Replacement of the *B. subtilis citC* gene by an *S. mutans* gene that expresses an NADH-producing ICDH completely restored sporulation to wild-type levels.

No single physiological condition or single suppressing mutation was able to restore sporulation completely to the *citC* mutant. Although addition of buffer, divalent cations, or certain metabolites to DS medium increased the fraction of the *citC* mutant population that survived and sporulated, it did not allow the vast majority of the population to advance beyond the original stage of blockage in the developmental program. Only the combination of reduction in citrate synthesis caused by a *citZ* mutation and alteration of the Spo0A transcription factor by the *sof-1* mutation was able to restore late *spo* gene expression to the *citC* mutant. These results have two implications. First, the defects caused by the absence of Krebs cycle enzymes are manifold and affect multiple stages of sporulation. Even when early blockages are overcome by alterations in the environment or by a suppressing mutation, the cells are still blocked at a late stage of sporulation. Although neither energy nor reducing power appears to be limiting at early stages, one or the other may eventually become limiting as mutant cells try to complete the sporulation process. Second, the cell population is heterogeneous in its response to the additions, an idea consistent with a mechanism in which threshold concentrations

of key metabolites determine whether any given cell will get past its genetically determined stage of blockage (5).

The mechanism of the synergistic suppression of the *citC* defect by mutations in *citZ* and *spo0A* (*sof-1*) is unclear. Since the *sof-1* mutation by itself has no suppressive effect and since the *citZ* mutation is epistatic to the *citC* mutation, the most likely explanation is that an alteration in the Spo0A phosphorelay allows the need for CitZ to be bypassed. The phenotype of a citrate synthase mutant is to be blocked at morphological stage III (23), but expression of even early classes of *spo* genes is delayed (49). Perhaps the *sof-1* mutation, which allows activation of Spo0A independently of the phosphorelay and normal developmental signals, overcomes a timing defect caused by the *citZ* mutation. In combination with a reduction in citrate synthesis and accumulation, the deleterious effects of the absence of ICDH are largely overcome.

In the accompanying paper (36), we show that loss-of-function mutations in the *spoVG* gene also cause a partial bypass of the *citC* mutant phenotype. The suppressive effect of a *spoVG* mutation is enhanced by deletion of *citZ*, especially in combination with a *sof-1* mutation. SpoVG is suspected to affect asymmetric septation by altering the expression or inhibiting the activity of a Spo0A-dependent gene.

Interestingly, mutants blocked in each of the first three steps in the Krebs cycle have different sporulation phenotypes. A *citA citZ* mutant, deficient in both citrate synthases, proceeds as far as stage III in sporulation (23, 49), while an aconitase (*citB*) null mutant is blocked at stage 0 (8). The citrate synthase mutant makes no citrate, and the pH of its medium (7.5) is considerably higher than that of the other two mutants, consistent with the idea that asymmetric septation and other early stages of sporulation are very pH sensitive and divalent cation dependent. In fact, the aconitase mutant may have the earliest block because it accumulates the greatest amount of extracellular citrate (8), and its extracellular pH is even lower (by about 0.4 pH unit) than that of the ICDH mutant (37). Not surprisingly, citrate synthase mutations are epistatic to both aconitase and ICDH mutations (reference 8 and this work).

It was previously reported that a mutant (Δ*citA*::*neo* Δ*citZ471 citC7*) lacking the major and minor citrate synthases and ICDH was blocked in sporulation at stage 0 (22). In the present study, we examined a strain (Δ*citA*::*neo* Δ*citZC*::*spc*) lacking the same three enzymes but created with different mutations and found that its sporulation phenotype was different than that of the previous strain. Cells of the newer strain were able to express Spo0A-phosphate-, σ^F- and σ^E-dependent genes. We assume that the specific mutations carried by the previous strain caused an unusual phenotype or that the strain had acquired an unrecognized, additional mutation that caused a block at stage 0.

The multiple deleterious changes in cellular physiology caused by the absence of ICDH activity provide compelling reasons for regulating synthesis of this enzyme during sporulation. The fact that the *citC* gene is transcribed from its own promoter as well as from the *citZCH* operon promoter (25) may allow the cell to adjust *citC* expression to the specific environmental conditions in which cells find themselves.

ACKNOWLEDGMENTS

We thank D. Cvitkovitch and P. Stragier for making plasmids and strains available, S. Jin for construction of strain SJB231, B. Belitsky for discussions and criticism of the manuscript, D. de Mendoza for advice on fatty acid supplementation, and A. Brown-Cormier for assistance with electron microscopy.

This work was supported in part by research grants from the U.S. Public Health Service to A.L.S. (GM42219) and from the U.S. National Science Foundation to T.C. (MCB-9723593) and to T.M.H. (MCB-9723091).

REFERENCES

1. **Arigoni, F., K. Pogliano, C. D. Webb, P. Stragier, and R. Losick.** 1995. Localization of protein implicated in establishment of cell type to sites of asymmetric division. Science **270:**637–640.
2. **Belitsky, B. R., M. C. U. Gustafsson, A. L. Sonenshein, and C. V. Wachenfeldt.** 1997. An *lrp*-like gene of *Bacillus subtilis* involved in branched-chain amino acid transport. J. Bacteriol. **179:**5448–5457.
3. **Belitsky, B. R.** Personal communication.
4. **Bhattacharya, M., L. Fuhrman, A. Ingram, K. W. Nickerson, and T. Conway.** 1995. Single-run separation and detection of multiple metabolic intermediates by anion-exchange high-performance liquid chromatography and application to cell pool extracts prepared from *Escherichia coli*. Anal. Biochem. **232:**98–106.
5. **Chung, J. D., G. Stephanopoulos, K. Ireton, and A. D. Grossman.** 1994. Gene expression in single cells of *Bacillus subtilis*: evidence that a threshold mechanism controls the initiation of sporulation. J. Bacteriol. **176:**1977–1984.
6. **Cosby, W. M., and P. Zuber.** 1997. Regulation of *Bacillus subtilis* σ^H (Spo0H) and AbrB in response to changes in external pH. J. Bacteriol. **179:**6778–6787.
7. **Cosby, W. M., D. Vollenbroich, O. H. Lee, and P. Zuber.** 1998. Altered *srf* expression in *Bacillus subtilis* resulting from changes in culture pH is dependent on the Spo0K oligopeptide permease and the ComQX system of extracellular control. J. Bacteriol. **180:**1438–1445.
8. **Craig, J. E., M. J. Ford, D. C. Blaydon, and A. L. Sonenshein.** 1997. A null mutation in the *Bacillus subtilis* aconitase gene causes a block in Spo0A-phosphate-dependent gene expression. J. Bacteriol. **179:**7351–7359.
9. **Cvitkovitch, D. G., J. A. Gutierrez, and A. S. Bleiweis.** 1997. Role of the citrate pathway in glutamate biosynthesis by *Streptococcus mutans*. J. Bacteriol. **179:**650–655.
10. **Duncan, L., S. Alper, F. Arigoni, R. Losick, and P. Stragier.** 1995. Activation of cell-specific transcription by a serine phosphatase at the site of asymmetric division. Science **270:**641–644.
11. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1–33.
12. **Fajardo-Cavazos, P., F. Tovar-Rojo, and P. Setlow.** 1991. Effect of promoter mutations and upstream deletions on the expression of genes coding for small, acid-soluble spore proteins of *Bacillus subtilis*. J. Bacteriol. **173:**2011–2016.
13. **Fisher, S.** Personal communication.
14. **Fortnagel, P., and E. Freese.** 1968. Inhibition of aconitase by chelation of transition metals causing inhibition of sporulation in *Bacillus subtilis*. J. Biol. Chem. **243:**5289–5295.
15. **Fortnagel, P., and E. Freese.** 1968. Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. J. Bacteriol. **95:**1431–1438.
16. **Fouet, A., and A. L. Sonenshein.** 1990. A target for carbon source-dependent negative regulation of the *citB* promoter of *Bacillus subtilis*. J. Bacteriol. **172:**835–844.
17. **Frandsen, N., and P. Stragier.** 1995. Identification and characterization of the *Bacillus subtilis spoIIP* locus. J. Bacteriol. **177:**716–722.

17a. **Freese, E., and U. Fortnagel.** 1969. Growth and sporulation of *Bacillus subtilis* mutants blocked in the pyruvate dehydrogenase complex. J. Bacteriol. **99:**745–756.

17b. **Freese, E., T. Ichikawa, Y. K. Oh, E. B. Freese, and C. Prasad.** 1974. Deficiencies or excesses of metabolites interfering with differentiation. Proc. Natl. Acad. Sci. USA **71:**4188–4193.

18. **Freese, E. B., and C. L. Marks.** 1973. Developmental block in citric acid cycle mutants of *Bacillus subtilis*. J. Bacteriol. **116:**1466–1468.
19. **Hederstedt, L.** 1993. The Krebs citric acid cycle, p. 181–197. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
20. **Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease.** 1989. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene **77:**51–59.
21. **Hoch, J. A., K. Trach, F. Kawamura, and H. Saito.** 1985. Identification of the transcriptional suppressor *sof-1* as an alteration in the *spo0A* protein. J. Bacteriol. **161:**552–555.
22. **Ireton, K., S. Jin, A. D. Grossman, and A. L. Sonenshein.** 1995. Krebs cycle function is required for activation of the Spo0A transcription factor in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **92:**2845–2849.
23. **Jin, S.** 1995. Ph.D. thesis. Tufts University, Boston, Mass.
24. **Jin, S., and A. L. Sonenshein.** 1994. Identification of two distinct *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**4669–4679.
25. **Jin, S., and A. L. Sonenshein.** 1994. Transcriptional regulation of *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**4680–4690.
26. **Jin, S., and A. L. Sonenshein.** 1996. Characterization of the major citrate synthase of *Bacillus subtilis*. J. Bacteriol. **178:**3658–3660.
27. **Jin, S., M. de Jesús-Berríos, and A. L. Sonenshein.** 1996. A *Bacillus subtilis* malate dehydrogenase gene. J. Bacteriol. **178:**560–563.

28. **Jin, S., P. A. Levin, K. Matsuno, A. D. Grossman, and A. L. Sonenshein.** 1997. Deletion of the *Bacillus subtilis* isocitrate dehydrogenase gene causes a block at stage I of sporulation. J. Bacteriol. **179:**4725–4732.

28a. **Khvorova, A., L. Zhang, M. L. Higgins, and P. J. Piggot.** 1998. The *spoIIE* locus is involved in the Spo0A-dependent switch in the location of FtsZ rings in *Bacillus subtilis*. J. Bacteriol. **180:**1256–1260.

29. **Krulwich, T. A., R. Agus, M. Schneier, and A. A. Guffanti.** 1985. Buffering capacity of bacilli that grow at different pH ranges. J. Bacteriol. **162:**768–772.

30. **Kunst, F., N. Ogasawara, I. Moszer, A. M. Albertini, G. Alloni, V. Azevedo, M. G. Bertero, P. Bessieres, A. Bolotin, S. Borchert, R. Borriss, L. Boursier, A. Brans, M. Braun, S. C. Brignell, S. Bron, S. Brouillet, C. V. Bruschi, B. Caldwell, V. Capuano, N. M. Carter, S. K. Choi, J. J. Codani, I. F. Connerton, A. Danchin, et al.** 1997. The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. Nature (London) **390:**249–256.

31. **Lakshmi, T. M., and R. B. Helling.** 1976. Selection for citrate synthase deficiency in *icd* mutants of *Escherichia coli*. J. Bacteriol. **127:**76–83.

32. **Levin, P. A., and R. Losick.** 1996. Transcription factor Spo0A switches the localization of the cell division protein FtsZ from a medial to a bipolar pattern in *Bacillus subtilis*. Genes Dev. **10:**478–488.

33. **Londoño-Vallejo, J.-A., C. Fréhel, and P. Stragier.** 1997. *spoIIQ*, a forespore-expressed gene required for engulfment in *Bacillus subtilis*. Mol. Microbiol. **24:**29–39.

34. **Magill, N. G., A. E. Cowan, D. E. Koppel, and P. Setlow.** 1994. The internal pH of the forespore compartment of *Bacillus megaterium* decreases by about 1 pH unit during sporulation. J. Bacteriol. **176:**2252–2258.

35. **Matsuno, K., S. Miller, D. C. LaPorte, and A. L. Sonenshein.** 1996. Biochemical studies of *B. subtilis* isocitrate dehydrogenase, abstr. II-3. *In* Abstracts of the 12th International Spores Conference.

36. **Matsuno, K., and A. L. Sonenshein.** 1999. Role of SpoVG in asymmetric septation in *Bacillus subtilis*. J. Bacteriol. **181:**3392–3401.

37. **Matsuno, K., and A. L. Sonenshein.** Unpublished data.

38. **Oh, Y. K., and E. Freese.** 1976. Manganese requirement of phosphoglycerate phosphomutase and its consequences for growth and sporulation of *Bacillus subtilis*. J. Bacteriol. **127:**739–746.

39. **Piggot, P. J., and J. G. Coote.** 1976. Genetic aspects of bacterial endospore formation. Bacteriol. Rev. **40:**908–962.

40. **Ramaley, R. F., and M. O. Hudock.** 1973. Purification and properties of isocitrate dehydrogenase (NADP) from *Thermus aquaticus* YT-1, *Bacillus subtilis*-168 and *Chlamydomonas reinhardti*-2. Biochim. Biophys. Acta **315:** 22–36.

41. **Rong, S., M. S. Rosenkrantz, and A. L. Sonenshein.** 1986. Transcriptional control of the *Bacillus subtilis spoIID* gene. J. Bacteriol. **165:**771–779.

42. **Rosenbluh, A., C. D. B. Banner, R. Losick, and P. C. Fitz-James.** 1981. Identification of a new developmental locus in *Bacillus subtilis* by construction of a deletion mutation in a cloned gene under sporulation control. J. Bacteriol. **148:**341–351.

43. **Rutberg, B., and J. A. Hoch.** 1970. Citric acid cycle: gene-enzyme relationships in *Bacillus subtilis*. J. Bacteriol. **104:**826–833.

44. **Sandman, K., R. Losick, and P. Youngman.** 1987. Genetic analysis of *Bacillus subtilis spo* mutations generated by Tn917-mediated insertional mutagenesis. Genetics **117:**603–617.

45. **Sandman, K., L. Kroos, S. Cutting, P. Youngman, and R. Losick.** 1988. Identification of the promoter for a spore coat protein gene in *Bacillus subtilis* and studies on the regulation of its induction at a late stage of sporulation. J. Mol. Biol. **200:**461–473.

46. **Segall, J., and R. Losick.** 1977. Cloned *Bacillus subtilis* DNA containing a gene that is activated early during sporulation. Cell **11:**751–761.

47. **Sonenshein, A. L.** 1989. Metabolic regulation of sporulation and other stationary-phase phenomena, p. 109–130. *In* I. Smith, R. A. Slepecky, and P. Setlow (ed.), Regulation of procaryotic development. American Society for Microbiology, Washington, D.C.

48. **Stragier, P., and R. Losick.** 1996. Molecular genetics of sporulation in *Bacillus subtilis*. Annu. Rev. Genet. **30:**297–341.

49. **Swift, K., and A. L. Sonenshein.** Unpublished data.

50. **Whatmore, A. M., and R. H. Reed.** 1990. Determination of turgor pressure in *Bacillus subtilis*: a possible role for K^+ in turgor pressure regulation. J. Gen. Microbiol. **136:**2521–2526.

51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

52. **Yansura, D. G., and D. J. Henner.** 1984. Use of *Escherichia coli lac* repressor and operator to control gene expression in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **81:**439–443.

53. **Yousten, A. A., and R. S. Hanson.** 1972. Sporulation of tricarboxylic acid cycle mutants of *Bacillus subtilis*. J. Bacteriol. **109:**886–894.

54. **Zuber, P., and R. Losick.** 1987. Role of AbrB in Spo0A- and Spo0B-dependent utilization of a sporulation promoter in *Bacillus subtilis*. J. Bacteriol. **169:**2223–2230.