JOURNAL OF BACTERIOLOGY, Dec. 1997, p. 7351–7359 Vol. 179, No. 23
0021-9193/97/$04.00+0

A Null Mutation in the *Bacillus subtilis* Aconitase Gene Causes a Block in Spo0A-Phosphate-Dependent Gene Expression

JANE E. CRAIG, MELANIE J. FORD,† DIANA C. BLAYDON,‡ AND ABRAHAM L. SONENSHEIN*

Department of Molecular Biology and Microbiology, Tufts University School of Medicine, Boston, Massachusetts 02111

Received 23 June 1997/Accepted 22 September 1997

The *citB* gene of *Bacillus subtilis* encodes aconitase, the enzyme of the Krebs citric acid cycle, which is responsible for the interconversion of citrate and isocitrate. A *B. subtilis* strain with an insertion mutation in the *citB* gene was devoid of aconitase activity and aconitase protein, required glutamate for growth in minimal medium, and was unable to sporulate efficiently in nutrient broth sporulation medium. Mutant cells failed to form the asymmetric septum characteristic of sporulating cells and were defective in transcription of the earliest-expressed *spo* genes, that is, the genes dependent on the Spo0A phosphorelay. However, this early block in sporulation was partially overcome when cells of the *citB* mutant were induced to sporulate by resuspension in a poor medium. Accumulation of citrate in the mutant cells or in their culture fluid may be responsible for the early block, possibly because citrate can chelate divalent cations needed for the activity of the phosphorelay.

Aconitase catalyzes the reversible isomerization of citrate and isocitrate via *cis*-aconitate in the Krebs citric acid cycle. In *Bacillus subtilis*, the enzyme is a monomeric iron-sulfur protein encoded by the *citB* gene (6). The primary sequence of aconitase (42) exhibits high similarity to aconitases from a wide variety of organisms (43) and to a family of iron response element binding proteins, which are involved in regulating iron homeostasis in many eukaryotic cells (16, 45).

When cells of *B. subtilis* begin to exhaust a complex medium and initiate sporulation, their aconitase activity increases sharply and decreases again by the second hour of stationary phase (7). In addition, it has been known for some time that mutants of *B. subtilis* lacking aconitase activity exhibit a sporulation defect (11, 54), suggesting a close link between expression of *citB* and sporulation.

The key step to controlling the initiation of sporulation is the phosphorylation of the regulatory protein Spo0A (14, 18). The phosphorylated form of this protein (Spo0A~P) is responsible for the transcriptional activation of key sporulation-specific genes, including those of the *spoIIA*, *spoIIG*, and *spoIIE* operons (40, 46, 47, 53). Spo0A~P also represses the transcription of *abrB*, which itself encodes a repressor of several genes involved in sporulation (39, 50). Phosphorylation of Spo0A occurs via an intricate phosphorelay system in which phosphate is transferred in a pathway from histidine protein kinases through intermediate phosphate carriers (2). The multiple steps of this pathway can be influenced by many factors, including nutrition (17, 19), culture density (19, 41), and DNA replication state (20). In this way, the phosphorelay allows various inputs from internal and external environmental sources to modulate the flux of phosphate through the pathway and thus control the decision to sporulate (14, 18).

The aim of this work was to construct a *citB* null mutant of *B. subtilis* in order to study more precisely the role of aconitase in the sporulation process. We found that when a *citB* mutant was grown in nutrient broth sporulation medium, there was a block in sporulation at stage 0. A mutation in *spo0A* that bypasses the need for the phosphorelay (17), allowing Spo0A to be phosphorylated by a different mechanism (29, 31), allows the *citB* mutant to proceed past the stage 0 block, indicating that the *citB* mutation interferes with the activity of the phosphorelay. In addition, when induced to sporulate by resuspension in a poor growth medium, the *citB* mutant was able to proceed to stage III in the sporulation pathway. This difference in phenotype could be attributed to accumulation of a sporulation inhibitory compound, probably citrate, by the *citB* mutant cells.

MATERIALS AND METHODS

Bacterial strains. All the bacterial strains used in this study are listed in Table 1.

DNA manipulations, transformations, and culture media. Plasmid DNA was isolated from *Escherichia coli* by a modification of the method of He et al. (15). Cleavage by restriction endonucleases, ligation, agarose gel electrophoresis, and Southern blot analyses were as described previously (4). DNA fragments were recovered from agarose with a GeneClean kit (Bio 101). Radiolabelled DNA probes were generated by using a DECAprime DNA labelling kit (Ambion Inc.). Preparation of electroporation-competent *E. coli* cells and transformation by electroporation with a Gene Pulser apparatus (Bio-Rad Laboratories) were as described by Dower et al. (9). Luria-Bertani medium (35) containing 50 μg of ampicillin per ml (when necessary) was used for growth of *E. coli*. Competent *B. subtilis* cells were prepared and transformed by the technique of Dubnau and Davidoff-Abelson (10). DS medium (13) containing 100 μg of spectinomycin per ml, 15 μg of tetracycline per ml, or 2.5 μg of chloramphenicol per ml (when necessary) was used for growth of *B. subtilis*. TSS minimal medium (13) supplemented with phenylalanine (0.004%, wt/vol) and tryptophan (0.004%, wt/vol) and with or without 0.2% (wt/vol) glutamate was used to test glutamate auxotrophy. The method used for induction of sporulation by resuspension in poor medium was essentially that of Sterlini and Mandelstam (49), except that the cells were resuspended in sporulation medium at an optical density at 600 nm of 0.6 to 0.7.

Construction of a mutant with an insertion mutation in *citB*. A 295-bp *Pst*I-*Bgl*II fragment from pMR41 containing codons 8 to 106 of the *citB* gene (Fig. 1) (44) was inserted into pJPM1 (Amp^r Cam^r) (36) cleaved with *Pst*I and *Bam*HI. The resulting plasmid (pDCB1) was integrated into the *B. subtilis* JH642 chromosome, selecting for chloramphenicol resistance. Chromosomal DNA from this strain was isolated, digested with *Sma*I, and religated under dilute conditions to favor intramolecular ligation. Ligated DNA was used to transform electrocompetent cells of *E. coli* JM107 to Amp^r. Plasmid DNA (pDCB2) isolated from a resulting transformant was found (by sequencing) to contain 1,478 bp corresponding to codons 8 to 500 of the *citB* gene. To construct a *citB* mutation,

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

† Present address: Department of Experimental Pathology, Medical School, Guy's Hospital, London SEI 9RT, England.

‡ Present address: Molecular Genetics Unit, Institute of Child Health, London WC1N 1EH, England.

TABLE 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
E. coli		
JM107	*endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac-proAB*)[F′ *traD36 proAB lacI*^{q} *lacZ*Δ*M15*]	Laboratory stock
B. subtilis		
AG918	*trpC2 pheA1 sof-1 cat*	A. Grossman
BMF9	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoIIA′-lacZ*) *cat trpC2 pheA1*	JH642 × SL4334 DNA
BMF13	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoIIG′-lacZ*) *cat trpC2 pheA1*	JH642 × EU8743 DNA
JCB36	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoIIA′-lacZ*) *cat trpC2 pheA1* Ω*citB*::*spc*	MAB160 × SL4334 DNA
JCB37	Φ(*spoIID′-lacZ*) *cat trpC2 pheA1* Ω*citB*::*spc*	MAB160 × SJB225 DNA
JCB38	Φ(*spoIIQ′-lacZ*) *trpC2 pheA1* Ω*citB*::*spc*	MAB160 × MO2051 DNA
JCB39	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoIIG′-lacZ*) *cat trpC2 pheA1* Ω*citB*::*spc*	MAB160 × EU8743 DNA
JCB43	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoVG′-lacZ*) *cat trpC2 pheA1* Ω*citB*::*spc*	MAB160 × ZB309 DNA
JCB50	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoVG′-lacZ*) *cat trpC2 pheA1*	JH642 × ZB309 DNA
JCB51	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ[*spoVG(42)′-lacZ*] *cat trpC2 pheA1*	JH642 × ZB456 DNA
JCB58	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ[*spoVG(42)′-lacZ*] *cat trpC2 pheA1* Ω*citB*::*spc*	MAB160 × ZB456 DNA
JCB61	*pheA1* Δ*citA*::*neo* Δ*citZ471* Ω*citB*::*spc*	SJB67 × MAB160 DNA
JCB63	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*cotA′-lacZ*) *tet trpC2 pheA1* Ω*citB*::*spc*	MAB160 × SC432 DNA
JCB64	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*cotA′-lacZ*) *tet trpC2 pheA1*	JH642 × SC432 DNA
JCB65	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*cotA′-lacZ*) *tet pheA1* Δ*citA*::*neo* Δ*citZ471* Ω*citB*::*spc*	JCB61 × SC432 DNA
JCB66	Φ(*spoIIQ′-lacZ*) *pheA1* Δ*citA*::*neo* Δ*citZ471* Ω*citB*::*spc*	JCB61 × MO2051 DNA
JCB67	Φ(*spoIID′-lacZ*) *cat pheA1* Δ*citA*::*neo* Δ*citZ471* Ω*citB*::*spc*	JCB61 × SJB225 DNA
JCB68	Φ(*sspB′-lacZ*) *cat pheA1* Δ*citA*::*neo* Δ*citZ471* Ω*citB*::*spc*	JCB61 × SJB227 DNA
JCB69	Φ(*sspB′-lacZ*) *cat trpC2 pheA1* Ω*citB*::*spc*	MAB160 × SJB227 DNA
JCB72	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoIIA′-lacZ*) *cat sof-1 cat*	SJB2192 × SL4334 DNA
JCB73	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoIIA′-lacZ*) *cat sof-1 cat* Ω*citB*::*spc*	JCB72 × MAB160 DNA
JH642	*trpC2 pheA1*	J. Hoch
MAB160	*trpC2 pheA1* Ω*citB*::*spc*	JH642 × pMEL1
EU8743	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoIIG′-lacZ*) *cat*	27
SL4334	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoIIA′-lacZ*) *cat*	P. Piggot
MO2051	Φ(*spoIIQ′-lacZ*) *trpC2 pheA1*	33
SC432	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*cotA′-lacZ*) *cat*::*tet*	5
SJB67	*pheA1* Δ*citA*::*neo* Δ*citZ471*	23
SJB219	Δ*citC*::*spc trpC2 pheA1*	25
SJB2192	Δ*citC*::*spc trpC2 pheA1 sof-1 cat*	SJB219 × AG918 DNA
SJB225	Φ(*spoIID′-lacZ*) *cat trpC2 pheA1*	24
SJB227	Φ(*sspB′-lacZ*) *cat trpC2 pheA1*	24
ZB309	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ(*spoVG′-lacZ*) *cat*	55
ZB456	SPβ*c2* Δ2::Tn*917*::pSK10Δ6::Φ[*spoVG(42)′-lacZ*] *cat*	55

plasmid pDCB2 was digested at the unique *Bgl*II site within the *citB* fragment and a spectinomycin resistance cassette from pJL74 (31) was inserted after unpaired ends were filled in by treatment with *E. coli* DNA polymerase I Klenow fragment. The resulting plasmid (pMEL1) was used by M. Nakano to transform wild-type *B. subtilis* JH642 cells, selecting for spectinomycin resistance and screening for chloramphenicol sensitivity. A double-crossover recombination event in one of the Spc^r Cml^s strains, called MAB160, was confirmed by Southern blot analysis (data not shown).

Enzyme assays. Cells for aconitase assays were grown in 100 ml of DS medium until the beginning of the stationary phase, harvested, and washed with a buffer containing 20 mM Tris-citrate (pH 7.35), 150 mM KCl, and 0.5 mM phenylmethylsulfonyl fluoride. The resulting pellets were frozen rapidly in dry ice and stored

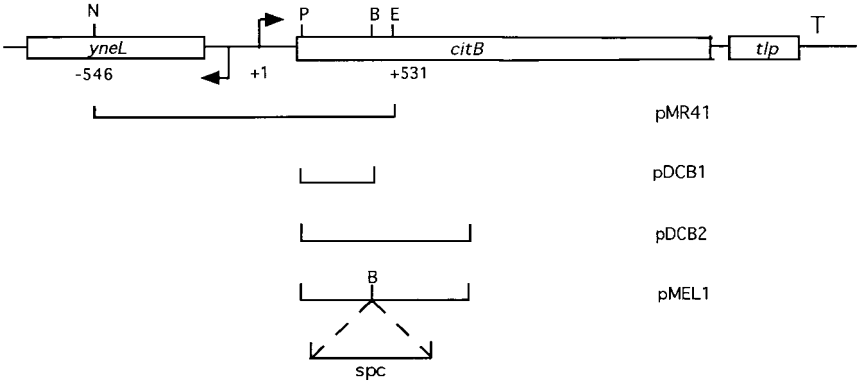

FIG. 1. Construction of a *citB* insertion mutant. The top line represents *B. subtilis* chromosomal DNA as deduced from the sequences given in references 6 and 42. The initiation sites and directions of transcription for *citB* and *yneL* are indicated by arrows. A putative transcription terminator is indicated by T. Restriction sites: B, *Bgl*II; E, *Eco*RI; P, *Pst*I; N, *Nde*I. As detailed in Materials and Methods, a fragment containing a *spc* gene was inserted at the *Bgl*II site in pDCB2 to create pMEL1. pMR41 is a pBR325 derivative (44). pDCB1, pDCB2, and pMEL1 are all derivatives of pJPM1 (36).

TABLE 2. Sporulation frequencies of *B. subtilis* strains[a]

Strain	Relevant genotype	Total titer at T_{20}[b]	Spore titer at T_{20}[b,c]	Sporulation efficiency[d]	Relative sporulation frequency[e]
JH642	*citB*$^+$	4.5×10^8	2.6×10^8	0.62	1.0
MAB160	*citB*::*spc*	4.4×10^6	8.8×10^2	2.2×10^{-4}	3.3×10^{-6}
JCB61	Δ*citA*::*neo* Δ*citZ471 citB*::*spc*	9.2×10^7	9.0×10^4	9.8×10^{-4}	3.5×10^{-4}
SJB67	Δ*citA*::*neo* Δ*citZ471*	9.0×10^7	5.5×10^5	6.1×10^{-3}	2.1×10^{-3}
JH642 in SM[f]	*citB*$^+$	3.1×10^8	1.7×10^8	0.55	1.0
MAB160 in SM	*citB*::*spc*	4.4×10^7	5.0×10^5	1.1×10^{-2}	2.9×10^{-3}

[a] Unless indicated otherwise, cells were grown in DS medium until 20 h after the end of the exponential growth phase and subjected to titer determination before and after heating to 80°C for 10 min.
[b] Titers are given in CFU per milliliter.
[c] Titer after being heated to 80°C for 10 min.
[d] The sporulation efficiency is the titer of heat-resistant cells divided by the total cell titer.
[e] The relative sporulation frequency is the spore production per milliliter relative to that of a wild-type culture under the same growth conditions.
[f] Cells were induced to sporulate by the method of Sterlini and Mandelstam (49).

overnight at −80°C. Cell extracts were prepared by resuspending the thawed pellet in 2 ml of the same buffer immediately prior to sonication (3 min total, with 2-s pulses and 2-s rests). Cell debris was eliminated by centrifugation (10 min at 12,000 × *g* and 4°C), and the supernatant fluid was used for the enzyme assay. A 5-μl sample of crude cell extract was mixed with 20 mM D,L-isocitrate and 50 mM Tris (pH 8.0), and the absorbance was monitored at 240 nm. One unit of aconitase activity is defined as a change in absorbance of 0.0033 (production of 1.0 nmol of *cis*-aconitate per ml) per min. Protein concentrations were determined by the Bio-Rad Coomassie blue assay with bovine serum albumin as the standard. β-Galactosidase enzyme assays were carried out as described elsewhere (1).

Assay of spore formation. Heat resistance was measured about 20 h after the end of the exponential phase (T_{20}) for all cultures. Samples were heated at 80°C for 10 min, and serial dilutions were plated on DS medium containing the appropriate antibiotic.

Electron microscopy. Cells were grown in DS medium to different time points of the postexponential phase. Samples (10 ml) were harvested, washed with 5 ml of 50 mM Tris (pH 8.0), resuspended in 1% freshly prepared glutaraldehyde in 100 mM sodium carbonate buffer, and stored at 4°C overnight. Osmification, NH_4Cl treatment, dehydration by repeated ethanol washes, Reynolds lead staining, Formvar coating, and sectioning were carried out by A. Brown-Cormier (Tufts University Electron Microscopy Facility).

Assay of citrate. Cultures of wild-type and mutant cells in DS medium were separated by centrifugation (15,000 × *g* for 5 min) into supernatant fluid and pellet fractions. Supernatant fluids were adjusted to pH 8.0 by the addition of KOH and assayed for their citric acid content with a kit obtained from Boehringer-Mannheim. The principle of the assay is the conversion of citrate to acetate and oxaloacetate by citrate lyase, coupled to reduction of oxaloacete by malate dehydrogenase with concomitant oxidation of NADH, measured as a loss of absorbance at 340 nm. Pelleted cells from 25-ml cultures were harvested, washed in 0.1 M Tris-HCl (pH 8.0), resuspended in 2 ml of Tris-HCl (pH 8.0), and broken by pulsed sonication for 3 min. After centrifugation, the supernatant fluid was adjusted to pH 8, if necessary, before the samples were assayed for citrate as above. Protein concentrations in the extracts were determined with the Bio-Rad Coomassie blue reagent.

Aconitase purification and polyclonal antibody production. Aconitase was purified by the method of Dingman et al. (7). About 0.5 mg of gel-purified protein was used (by D. Acheson) for polyclonal antiserum production in mice. Mouse serum immunoglobulin G was purified through a protein G-Sepharose column.

Polyacrylamide gel electrophoresis, Western blotting, and immunodetection. Cell extracts prepared as described for the aconitase assay were separated by electrophoresis in sodium dodecyl sulfate–polyacrylamide gels (30). Samples in Laemmli sample buffer were boiled for 2 min before being loaded onto the gel. Procedures for Western transfer and the detection of bound antisera by alkaline phosphatase staining were as described elsewhere (28, 52).

RESULTS

Phenotype of a *citB* null mutant. A *citB* insertion mutant (MAB160) was constructed as described in Materials and Methods. MAB160 exhibited an absolute requirement for glutamate during growth on glucose minimal medium, indicating that *citB* encodes the only major aconitase functioning during normal growth in *B. subtilis*. As expected, the aconitase enzyme activity of the *citB* mutant at the onset of stationary phase (T_0) was negligible (≤10 U per mg) compared to that in wild-type cells harvested at the same time point (354 U per mg). The absence of aconitase protein in MAB160 was confirmed by immunoblot analysis (data not shown). The mutation in *citB* caused a drop in cell viability with a 100-fold-lower yield than in a wild-type culture, and a very severe sporulation defect was also observed, with only 1 in 5,000 viable cells forming a spore and a total spore yield 300,000-fold lower than in a wild-type culture (Table 2). A deletion-insertion mutation in *tlp*, the gene immediately downstream of *citB*, caused no discernible phenotype, showing that the properties of the *citB* mutant are not due to polarity on downstream genes.

Electron microscopic examination of MAB160. To identify the morphological stage at which sporulation was blocked in strain MAB160, thin sections of stationary-phase cells were examined by electron microscopy. The results are shown in Fig. 2. With the *citB* mutant, no evidence for the appearance of the asymmetric septum or any spore-like structures could be seen even 7 h after the onset of the stationary phase (T_7). By comparison, the majority of cells in a wild-type population showed extensive progress in the developmental cycle by T_3. These results are in agreement with the earlier studies of Yousten and Hanson (54), who showed that a genetically undefined mutant defective in aconitase activity is blocked at stage 0 to I of sporulation.

Expression of sporulation genes in MAB160. To determine the stage at which sporulation is blocked in strain MAB160, the levels of expression of several genes normally expressed at characteristic developmental stages were measured. Fusions of the sporulation gene promoters to the *E. coli lacZ* gene were introduced in single copy into the chromosome of MAB160, and the expression of each fusion was monitored by measuring β-galactosidase production.

Transcription of the *spoIIA* and *spoIIG* operons is positively regulated by Spo0A~P (40, 46, 47), and these genes are transcribed during the first hour of the stationary phase in the preseptational cell. In the *citB* mutant, the expression of these genes was clearly reduced or greatly delayed compared to expression in wild-type cells (Fig. 3A and B). *spoVG* transcription is also induced at the onset of the stationary phase. Its induction depends on the ability of Spo0A~P to repress transcription of *abrB* (55). (AbrB represses *spoVG* transcription [39].) As can be seen from Fig. 3C, *spoVG-lacZ* expression was induced to a significantly lower level in the *citB* mutant. To discover if this effect is AbrB dependent, a second *spoVG-lacZ* fusion [*spoVG(42)-lacZ*] was introduced into the chromosome of MAB160. This fusion contains a single base change just upstream of the *spoVG* −35 promoter region that allows *spoVG(42)* expression to be independent of AbrB (55). In

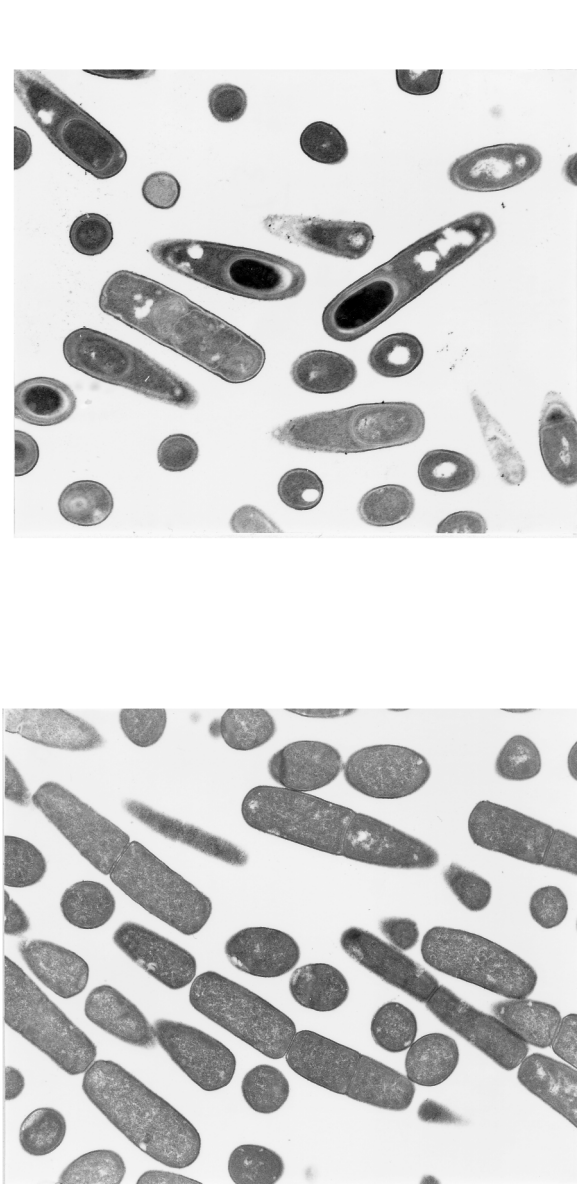

FIG. 2. Electron microscopic analysis. Samples (prepared as described in Materials and Methods) of strains JH642 (*citB*$^+$) harvested 3 h after the onset of the stationary phase (T_3) (A) and MAB160 harvested at T_7 (B) were examined by electron microscopy.

MAB160, *spoVG(42)-lacZ* expression was not repressed and, in fact, reached a higher level than in wild-type cells (Fig. 3D). The implication of these results is that *citB* mutant cells have a lower level of Spo0A~P than do wild-type cells at the equivalent growth stage.

Following septation, transcription in the forespore and mother cell is directed initially by RNA polymerase containing the sigma factors σ^F and σ^E. Genes requiring these sigma factors for transcription include *spoIIQ* and *spoIID*, respectively. As expected, in strain MAB160, the expression of *spoIID-lacZ* and *spoIIQ-lacZ* fusions was almost completely blocked (data not shown).

The *sof-1* mutation in *spo0A* suppresses the stage 0 block in MAB160. The *sof-1* mutation in *spo0A* allows the gene product to be phosphorylated by a mechanism that is independent of the phosphorelay (17, 29, 31). Thus, if the block in expression of early sporulation genes in the *citB* mutant were due to a failure to activate the phosphorelay, this block would be bypassed in a *sof-1* mutant strain. In fact, in a *citB sof-1* strain, expression of *spoIIA-lacZ* reached a higher level than in wild-type cells (Fig. 4), indicating that the *citB* mutant is unable to express the *spoIIA* operon because it cannot activate Spo0A by the normal phosphorylation pathway. However, the sporulation efficiency of the *citB sof-1* double mutant was not greatly increased compared to that of the *citB* mutant alone (data not shown), suggesting that the sporulation pathway is still blocked at a later stage.

Expression of sporulation genes in MAB160 in resuspension medium. It has been previously suggested that the inability of an aconitase mutant to sporulate is due, in part, to the presence of an inhibitory substance(s) in the growth medium (54). Other possible reasons for the lack of sporulation in a *citB* mutant include the inability to produce a signal required during the early stages of sporulation and the failure to produce enough energy for sporulation. To study these possibilities, we looked to see if sporulation could be induced in the *citB* mutant by resuspension of exponentially growing cells in a nutrient-limited medium, essentially by the method of Sterlini and Mandelstam (49). The expression of *spoIIA-lacZ*, *spoIID-lacZ*, *sspB-lacZ*, and *cotA-lacZ* fusions was assayed in wild-type and MAB160 cells at various times following resuspension. SspB is found in the spore core, and its gene is transcribed by the σ^G form of RNA polymerase, typically starting 2.5 h after the onset of the stationary phase (48). *cotA* encodes a spore coat protein (8) and is typically expressed 3 to 4 h after the onset of the stationary phase under the control of RNA polymerase containing the sporulation-specific sigma factor σ^K. It can be seen from Fig. 5 that both *spoIIA* and *spoIID* were expressed in MAB160 to a level similar to that in wild-type cells. *sspB* and *cotA* were also expressed but at reduced levels compared to that in wild-type cells. The sporulation frequency of the *citB* mutant in this medium was around 100-fold higher than in DS medium but was still not restored to wild-type levels (Table 2). These results imply that the absence of aconitase does not necessarily interfere with the earliest stages of sporulation. In fact, it appears that, if the *citB* mutant is removed from its growth medium and resuspended in a nutritionally poorer medium, a larger fraction of the cell population can complete sporulation. Even cells that do not complete the process (i.e., the majority of the cells) can proceed to a later stage of sporulation than previously thought.

The results of the resuspension experiments suggest that when grown in DS medium, MAB160 accumulates a material that inhibits sporulation. Since aconitase catalyzes the reversible conversion of citrate to isocitrate, it is possible that in a *citB* mutant there is an overaccumulation of citrate in the cell or in the medium. We therefore measured the concentration of citrate in the growth medium and in extracts of mutant and wild-type cells. As shown in Fig. 6, there was no detectable citrate in the medium of wild-type cells at any time during exponential growth phase or stationary phase, but strain MAB160 began to accumulate citrate to very high levels as the cells reached the end of the exponential growth phase, coincident with the induction of citrate synthase (22). The maximal concentration reached (~2 mM) was at least 80 times higher than in wild-type cells. Extracts of mutant cells also contained a higher citrate concentration (12 μg/mg of protein) than did extracts of wild-type cells (5 μg/mg of protein), but the difference was only twofold. Given that citrate is thought to be the inducer of *citB* (7, 38), overaccumulation of citrate by strain MAB160 is consistent with the hyperexpression of a *citB-lacZ*

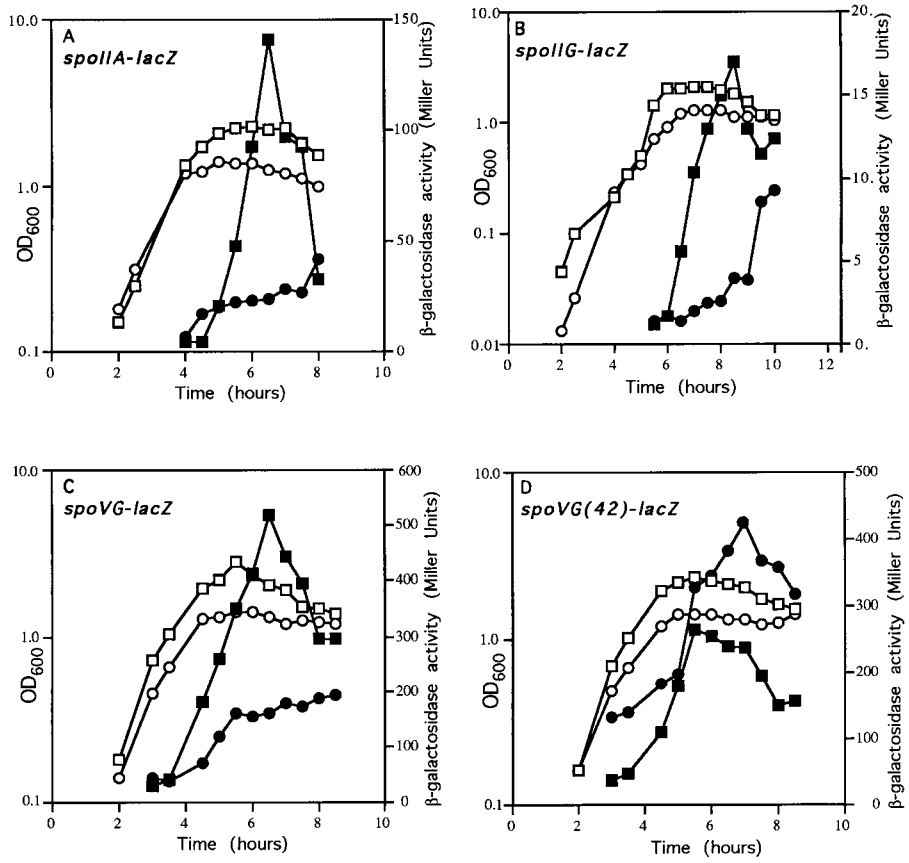

FIG. 3. Expression of early-stationary-phase genes in wild-type and *citB* mutant cells. Wild-type (squares) and *citB* mutant (circles) strains were grown in DS medium, and samples were taken at indicated times to monitor growth (open symbols) and β-galactosidase activity (solid symbols) (A) *spoIIA-lacZ* activity, strains BMF9 and JCB36; (B) *spoIIG-lacZ* activity, strains BMF13 and JCB39; (C) *spoVG-lacZ* activity, strains JCB43 and JCB50; (D) *spoVG(42)-lacZ* activity, strains JCB51 and JCB58. OD_{600}, optical density at 600 nm.

fusion in that strain compared to expression in wild-type cells (data not shown).

To show that citrate is at least one of the factors that causes an early block in sporulation in strain MAB160, we supplemented Sterlini-Mandelstam resuspension medium with citrate at various concentrations. Figure 7 shows that the addition of citrate inhibited the expression of a *spoIIA-lacZ* fusion in the wild-type strain and, to an even greater extent, in strain MAB160.

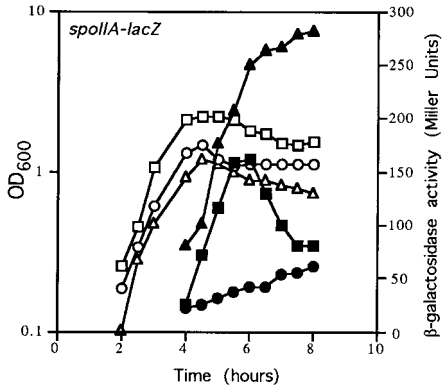

FIG. 4. Expression of *spoIIA-lacZ* in wild-type (BMF9), *citB* mutant (JCB36), and *citB sof-1* double-mutant (JCB73) cells. Wild-type (squares), *citB* mutant (circles), and *citB sof-1* double-mutant (triangles) strains were grown in DS medium, and samples were taken at the indicated times to monitor growth (open symbols) and β-galactosidase activity (solid symbols). OD_{600}, optical density at 600 nm.

Addition of divalent cations to DS medium increases the sporulation efficiency of MAB160. Citrate is a chelator of various divalent metal ions and could therefore have a deleterious effect when present in large quantities in a cell. Manganese and iron are known to be essential for sporulation (3, 12), and chelating agents are known to inhibit sporulation (12). To examine the possibility that the sporulation defect in the *citB* mutant was due to chelation of essential divalent cations, cells were grown in medium containing a 75-fold excess of $MnCl_2$ or $FeSO_4$ relative to standard DS medium. As can be seen in Table 3, the sporulation frequency of MAB160 increased significantly upon the addition of excess Mn^{2+} ions to the medium and to a lesser extent upon the addition of excess Fe^{2+} ions. Sporulation was still not restored to wild-type levels, however.

Effect of simultaneous blockage of citrate synthase and aconitase. If accumulation of citrate were responsible, at least in part, for the sporulation defect of MAB160, a mutant defective in both citrate synthase and aconitase might be less defective in sporulation than is MAB160. Citrate synthases in *B. subtilis* are encoded by two homologous genes, *citA* and *citZ* (23). A *citA citZ citB* triple mutant was constructed by transforming strain SJB67 (*citA citZ*) with MAB160 chromosomal

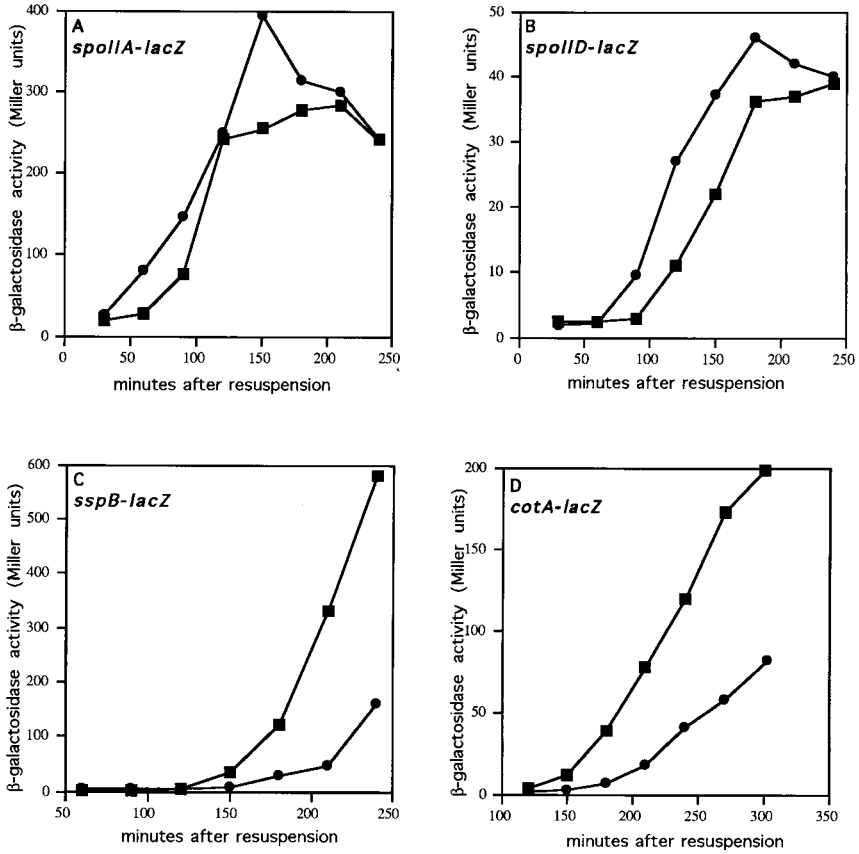

FIG. 5. Expression of sporulation genes in wild-type and *citB* mutant cells following resuspension in Sterlini-Mandelstam medium. Wild-type (squares) and *citB* mutant (circles) strains were grown initially in Sterlini-Mandelstam medium supplemented with 1% Casamino Acids. At mid-exponential phase, cells were harvested and resuspended in the same medium without Casamino Acids to give an OD_{600} of 0.6 to 0.7. Following resuspension, samples were taken for the β-galactosidase assay at the time intervals indicated. (A) *spoIIA-lacZ* expression, strains BMF9 and JCB36; (B) *spoIID-lacZ* expression, strains BMF4 and JCB37; (C) *sspB-lacZ* expression, strains SJB227 and JCB69; (D) *cotA-lacZ* expression, strains JCB64 and JCB63.

DNA and selecting for spectinomycin resistance. The resulting strain (JCB61) had a 10-fold-higher yield after overnight growth in DS medium than did the *citB* single mutant, and its sporulation frequency was 100-fold higher than that seen with the *citB* single mutant (Table 2). Strain JCB61 did not accumulate any detectable citrate in the medium (data not shown), and extracts of this strain had less than 5 μg of citrate/mg of protein.

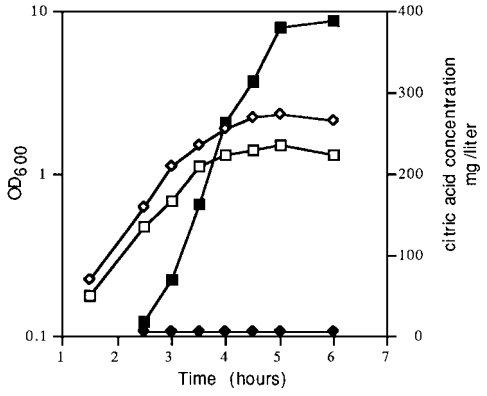

FIG. 6. Citrate accumulation in the culture fluid of wild-type (diamonds) and *citB* mutant (squares) cells. Wild-type and MAB160 strains were grown in DS medium. At the indicated times, 1-ml samples were subjected to centrifugation. The supernatant fluids were adjusted to pH 8.0 and assayed for citric acid content (solid symbols) as described under Materials and Methods. Growth curves are shown by open symbols. OD_{600}, optical density at 600 nm.

To examine the stage of sporulation blockage in JCB61, the expression of *spoIIA-lacZ*, *spoIID-lacZ*, *sspB-lacZ*, and *cotA-lacZ* fusions was assayed. There was no *sspB-lacZ* or *cotA-lacZ* expression in the triple mutant, but *spoIIQ* and *spoIID* were both expressed, albeit at a later time than in wild-type cells (Fig. 8).

DISCUSSION

We have shown that a *citB* null mutant of *B. subtilis* exhibits a severe early defect in sporulation when grown in nutrient broth sporulation medium. The expression of genes that depend on Spo0A~P for their induction (including *spoIIA*, *spoIIG*, and *spoVG*) is greatly reduced or delayed by the *citB* mutation, and no asymmetric septum appears. Thus, the block occurs at stage 0. Since the *sof-1* allele of *spo0A* restores *spoIIA-lacZ* expression to MAB160, it appears that the *citB* mutation affects in some way the activity of the phosphorelay.

The stage 0 block can also be overcome, allowing the mutant to proceed to a later stage of the sporulation pathway, if mutant cells are removed from their growth medium and resuspended in a nutrient-poor medium. This result could mean that the early block in sporulation is due to the accumulation of an inhibitory compound when the cells are grown in DS

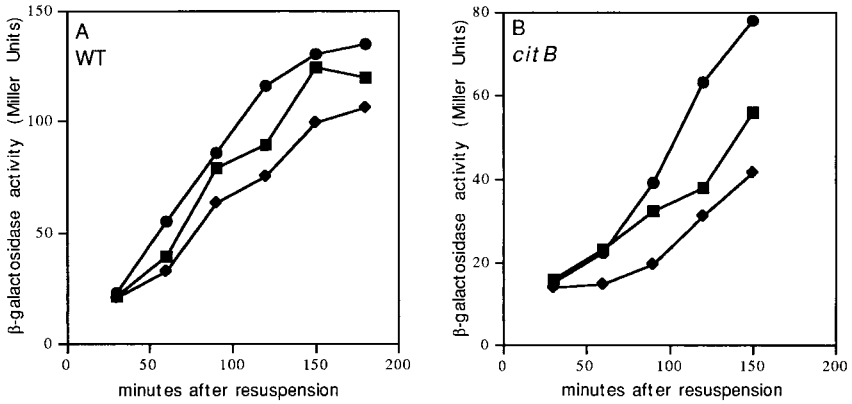

FIG. 7. Expression of *spoIIA-lacZ* in Sterlini-Mandelstam resuspension medium supplemented with citrate. Wild-type (WT) (BMF39) (A) and *citB* mutant (JCB36) (B) strains in the mid-exponential growth phase were resuspended in Sterlini-Mandelstam medium as described in the legend to Fig. 5. As indicated, the medium was supplemented with trisodium citrate dihydrate to give final concentrations of 0% (circles), 0.2% (squares), or 1% (diamonds).

medium. A likely candidate for such a factor is citrate, given that the *citB* mutant accumulates vastly more citrate than does a wild-type cell and that a *citA citZ citB* triple mutant proceeds to stage III.

The link between citrate accumulation and the early block in sporulation may be related to the ability of citrate to chelate divalent cations. When we added an excess of Mn^{2+} and Fe^{2+} ions to DS medium, we were able to increase the sporulation frequency of the *citB* mutant by up to 100-fold. Moreover, we have shown that when wild-type cells are grown in the absence of manganese, genes dependent on Spo0A~P are not expressed (51). This early arrest in wild-type cells depleted of manganese is reminiscent of the early sporulation defect seen with a *citB* mutant.

How manganese might influence the phosphorelay is unknown. A candidate target is phosphoglycerate phosphomutase, which catalyzes the interconversion of 3-phosphoglyceric acid (3-PGA) and 2-PGA in the glycolytic pathway and has a specific manganese requirement. In the absence of manganese, wild-type cells of *B. subtilis* stop growing at a lower than normal cell density, 3-PGA accumulates inside the cell, and sporulation is arrested before asymmetric septation (32, 37). No direct relationship between accumulation of 3-PGA and the activity of the Spo0A phosphorelay has been demonstrated, however. Ferrous ions do not produce as dramatic an effect as manganous ions, but they do allow a 10-fold increase in sporulation. The role of excess ferrous ions, in this context, may be to liberate manganous ions by displacing them from citrate, or they may also play an important role in sporulation but are just not transported into cells as efficiently as are manganous ions.

The citrate thought to be the inhibitory substance is produced intracellularly by the products of two citrate synthase genes (*citA* and *citZ*) (23). Cells that carry null mutations in *citA* and *citZ* as well as *citB* are able to proceed through stages I and II of sporulation but are unable to express *sspB-lacZ* or *cotA-lacZ* fusions, indicating blocks in σ^G-dependent and σ^K-dependent transcription. A *citA citZ* double mutant has a similar block in gene expression (24), and the results of our resuspension experiments indicate that the *citB* mutant also proceeds to this stage of the sporulation pathway when removed from the putative inhibitory compound(s). Thus, our results suggest that a *citB* mutant fails to sporulate for at least two reasons: it is unable to activate expression of Spo0A~P-dependent genes, and it fails to activate σ^G-dependent gene expression. The basis of this later block is unclear. It is known that activation of σ^G in the forespore requires completion of engulfment and a signal from the mother cell provided, at least in part, by the products of the *spoIIIA* operon (26, 34). How the lack of citrate synthase or aconitase influences these events is unknown.

In previous work, we found that a mutant defective in both citrate synthase and isocitrate dehydrogenase is blocked in the expression of Spo0A~P-dependent genes (21). Since such a mutant cannot make citrate from either pyruvate or 2-ketoglutarate, there must be more than one reason why tricarboxylic acid cycle mutants have early developmental blocks. *B. subtilis* cells normally induce citrate synthase and aconitase enzyme

TABLE 3. Effect of excess $FeSO_4$ or $MnCl_2$ on sporulation[a]

Strain	Supplement	Total titer at T_{20}[b]	Heat-resistant titer at T_{20}[b]	Sporulation efficiency[c]	Relative sporulation frequency[c]
JH642	None	7.1×10^8	6.1×10^8	0.86	1.0
	0.75 mM $MnCl_2$	5.0×10^8	2.2×10^8	0.44	1.0
	0.075 mM $FeSO_4$	6.0×10^8	4.2×10^8	0.70	1.0
MAB160	None	4.4×10^6	8.8×10^2	2.2×10^{-4}	1.4×10^{-6}
	0.75 mM $MnCl_2$	2.0×10^7	9.9×10^5	5.0×10^{-2}	4.5×10^{-3}
	0.075 mM $FeSO_4$	1.0×10^6	4.0×10^3	4.0×10^{-3}	9.5×10^{-6}

[a] Cells were grown in DS medium with or without supplementation of $MnCl_2$ or $FeSO_4$. The unsupplemented medium contains 0.01 mM $MnCl_2$ and 0.001 mM $FeSO_4$.

[b] Titers are given in CFU per milliliter.

[c] The sporulation efficiency and relative sporulation frequency were defined as in Table 2, footnotes *d* and *e*.

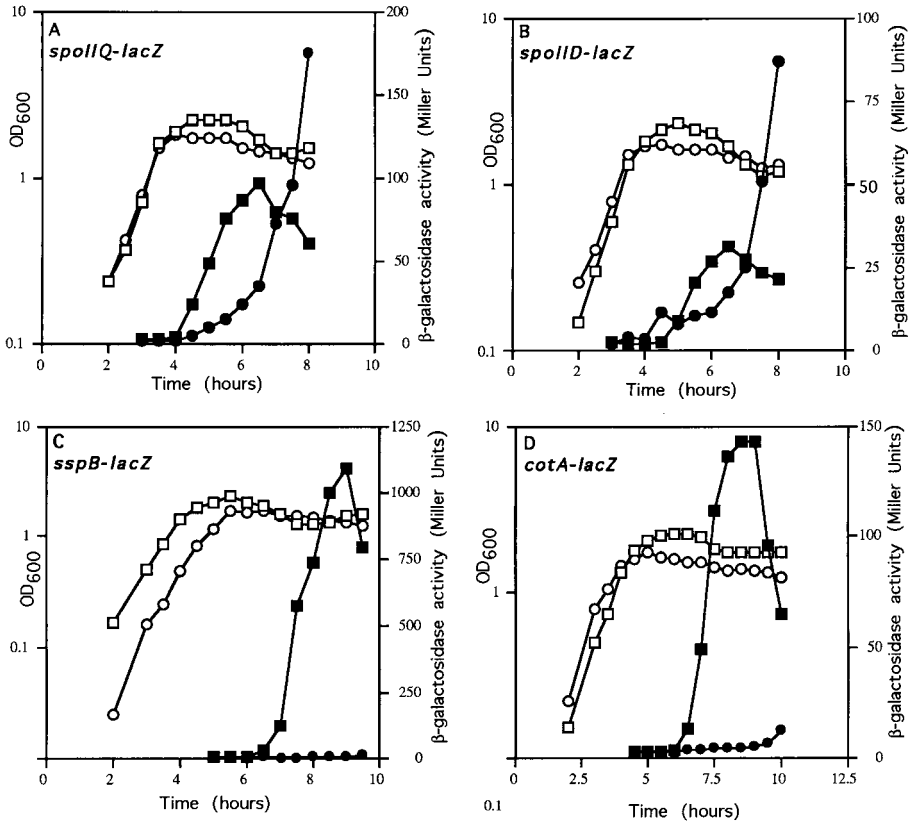

FIG. 8. Expression of sporulation genes in wild-type cells and *citA citZ citB* triple mutant. Wild-type (squares) and *citA citZ citB* mutant (circles) strains were grown in DS medium, and samples were taken at the indicated times to monitor growth (open symbols) and β-galactosidase activity (solid symbols). (A) *spoIIQ-lacZ* activity, strains BMF10 and JCB66; (B) *spoIID-lacZ* activity, strains JCB67 and BMF4; (C) *sspB-lacZ* activity, strains SJB227 and JCB68; (D) *cotA-lacZ* activity, strains JCB64 and JCB65. OD_{600}, optical density at 600 nm.

activities sequentially at a very early stage of sporulation. If the cells were to induce citrate synthase but not aconitase, however, as in the case of a *citB* mutant, sporulation would become blocked very early due to the accumulation of an inhibitory compound (citrate). Thus, induction of aconitase synthesis by citrate accumulation in wild-type cells appears to be a genetic coupling that is essential for sporulation.

ACKNOWLEDGMENTS

We thank M. Nakano for creating strain MAB160, M. Rose and K.-D. Entian for making sequence data available before publication, D. Acheson for help with antibody production, and K. Matsuno, B. Belitsky, and M. Malamy for helpful discussions.

This research was supported by grant GM42219 from the U.S. Public Health Service to A.L.S.

REFERENCES

1. **Belitsky, B. R., P. J. Janssen, and A. L. Sonenshein.** 1995. Sites required for GltC-dependent regulation of *Bacillus subtilis* glutamate synthase expression. J. Bacteriol. **177:**5686–5695.
2. **Burbulys, D., K. A. Trach, and J. A. Hoch.** 1991. Initiation of sporulation in *Bacillus subtilis* is controlled by a multicomponent phosphorelay. Cell **64:** 545–552.
3. **Charney, J., W. P. Fisher, and C. P. Hegarty.** 1951. Manganese as an essential element for sporulation in the genus *Bacillus*. J. Bacteriol. **62:**145–148.
4. **Craig, J. E., Z.-B. Zhang, and M. P. Gallagher.** 1994. Cloning of the *nupC* gene of *Escherichia coli* encoding a nucleoside transport system and identification of an adjacent insertion element, IS186. Mol. Microbiol. **11:**1159–1168.
5. **Cutting, S., V. Oke, A. Driks, R. Losick, S. Lu, and L. Kroos.** 1990. A forespore checkpoint for mother cell gene expression during development in *B. subtilis*. Cell **62:**239–250.
6. **Dingman, D. W., and A. L. Sonenshein.** 1987. Purification of aconitase from *Bacillus subtilis* and correlation of its N-terminal amino acid sequence with the sequence of the *citB* gene. J. Bacteriol. **169:**3062–3067.
7. **Dingman, D. W., M. S. Rosenkrantz, and A. L. Sonenshein.** 1987. Relationship between aconitase gene expression and sporulation in *Bacillus subtilis*. J. Bacteriol. **169:**3068–3075.
8. **Donovan, W., L. Zheng, K. Sandman, and R. Losick.** 1987. Genes encoding spore coat polypeptides from *Bacillus subtilis*. J. Mol. Biol. **196:**1–10.
9. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
10. **Dubnau, D., and R. Davidoff-Abelson.** 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. J. Mol. Biol. **56:**209–221.
11. **Fortnagel, P., and E. Freese.** 1968. Analysis of sporulation mutants. II. Mutants blocked in the citric acid cycle. J. Bacteriol. **95:**1431–1438.
12. **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.
13. **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.
14. **Grossman, A. D.** 1991. Integration of developmental signals and the initiation of sporulation in *Bacillus subtilis*. Cell **65:**5–8.
15. **He, M., M. A. Kaderdhai, I. Adcock, and B. M. Austen.** 1991. An improved and rapid procedure for isolating RNA-free *Escherichia coli* plasmid DNA. Genet. Anal. Tech. Applic. **8:**107–110.
16. **Hentze, M. W., and L. C. Kuhn.** 1996. Molecular control of vertebrate iron metabolism: mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc. Natl. Acad. Sci. USA **93:**8175–8182.
17. **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.
18. **Hoch, J. A.** 1993. Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. Annu. Rev. Microbiol. **47:**441–465.
19. **Ireton, K., D. Z. Rudner, K. J. Siranosian, and A. D. Grossman.** 1993. Integration of multiple developmental signals in *Bacillus subtilis* through the

Spo0A transcription factor. Genes Dev. **7:**283–294.
20. **Ireton, K., and A. D. Grossman.** 1994. A developmental checkpoint couples the initiation of sporulation to DNA replication in *Bacillus subtilis*. EMBO J. **13:**1566–1573.
21. **Ireton, K., S. Jin, A. Grossman, and A. L. Sonenshein.** 1995. Krebs cycle function is required for activation of the SpoOA transcription factor in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **92:**2845–2849.
22. **Jin, S., and A. L. Sonenshein.** 1994. Transcriptional regulation of *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**4680–4690.
23. **Jin, S., and A. L. Sonenshein.** 1994. Identification of two distinct *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**4669–4679.
24. **Jin, S.** 1995. Ph.D. thesis. Tufts University, Boston, Mass.
25. **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.
26. **Karmazyn-Campelli, C., C. Bonamy, B. Savelli, and P. Stragier.** 1989. Tandem genes encoding σ factors for consecutive steps of development in *Bacillus subtilis*. Genes Dev. **3:**150–157.
27. **Kenney, T. J., and C. P. Moran, Jr.** 1987. Organization and regulation of an operon that encodes a sporulation-essential sigma factor in *Bacillus subtilis*. J. Bacteriol. **169:**3329–3339.
28. **Knecht, D. A., and R. L. Dimond.** 1984. Visualisation of antigenic proteins on Western blots. Anal. Biochem. **136:**180–184.
29. **Kobayashi, K., K. Shoji, T. Shimizu, K. Nakano, T. Sato, and Y. Kobayashi.** 1995. Analysis of a suppressor mutation *ssb* (*kinC*) of *sur0B20* (*spo0A*) mutation in *Bacillus subtilis* reveals that *kinC* encodes a histidine protein kinase. J. Bacteriol. **177:**176–182.
30. **Laemmli, U. K.** 1970. Cleavage of structural proteins during assembly of bacteriophage T4. Nature **227:**680–685.
31. **LeDeaux, J. R., and A. D. Grossman.** 1995. Isolation and characterization of *kinC*, a gene that encodes a sensor kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. J. Bacteriol. **177:**166–175.
32. **Leyva-Vazquez, M. A., and P. Setlow.** 1994. Cloning and nucleotide sequences of the genes encoding triose phosphate isomerase, phosphoglycerate mutase, and enolase from *Bacillus subtilis*. J. Bacteriol. **176:**3903–3910.
33. **Londono-Vallejo, A., C. Frehel, and P. Stragier.** 1997. *spoIIQ*, a forespore-expressed gene required for engulfment in *Bacillus subtilis*. Mol. Microbiol. **24:**29–39.
34. **Mason, J. M., R. H. Hackett, and P. Setlow.** 1988. Regulation of expression of genes coding for small, acid-soluble proteins of *Bacillus subtilis* spores: studies using *lacZ* gene fusions. J. Bacteriol. **170:**239–244.
35. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Mueller, J. P., G. Bukusoglu, and A. L. Sonenshein.** 1992. Transcriptional regulation of *Bacillus subtilis* glucose starvation-inducible genes: control of *gsiA* by the ComP-ComA signal transduction system. J. Bacteriol. **174:**4361–4373.
37. **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.
38. **Ohne, M.** 1973. Regulation of aconitase synthesis in *Bacillus subtilis*: induction, feedback repression and catabolite repression. J. Bacteriol. **117:**1295–1305.
39. **Perego, M., G. B. Spiegelman, and J. A. Hoch.** 1988. Structure of the gene for the transition state regulator, *abrB*: regulator synthesis is controlled by the *spo0A* sporulation gene in *Bacillus subtilis*. Mol. Microbiol. **2:**689–699.
40. **Perego, M., J.-J. Wu, G. B. Spiegelman, and J. A. Hoch.** 1991. Mutational dissociation of the positive and negative regulatory properties of the Spo0A sporulation transcription factor of *Bacillus subtilis*. Gene **100:**207–212.
41. **Perego, M., and J. A. Hoch.** 1996. Cell-cell communication regulates the effects of protein aspartate phosphatases on the phosphorelay controlling development in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **93:**1549–1553.
42. **Rose, M., and K.-D. Entian.** 1996. Personal communication.
43. **Rose, M., and K.-D. Entian.** 1996. New genes in the 170° region of the *Bacillus subtilis* genome encode DNA gyrase subunits, a thioredoxin, a xylanase and an amino acid transporter. Microbiology **142:**3097–3101.
44. **Rosenkrantz, M. S., D. W. Dingman, and A. L. Sonenshein.** 1985. *Bacillus subtilis citB* gene is regulated synergistically by glucose and glutamine. J. Bacteriol. **164:**155–164.
45. **Rouault, T. A., and R. D. Klausner.** 1996. Iron-sulfur clusters as biosensors of oxidants and iron. Trends Biochem. Sci. **21:**174–177.
46. **Satola, S. W., P. A. Kirchman, and C. P. Moran, Jr.** 1991. Spo0A binds to a promoter used by the σ^A RNA polymerase during sporulation in *Bacillus subtilis*. Proc. Natl. Acad. Sci. USA **88:**4533–4537.
47. **Satola, S. W., J. M. Baldus, and C. P. Moran, Jr.** 1992. Binding of Spo0A stimulates *spoIIG* promoter activity in *Bacillus subtilis*. J. Bacteriol. **174:** 1448–1453.
48. **Setlow, P.** 1988. Small, acid-soluble spore proteins of *Bacillus* species: structure, synthesis, genetics, function, and degradation. Annu. Rev. Microbiol. **42:**319–338.
49. **Sterlini, J. M., and J. Mandelstam.** 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem. J. **113:**29–37.
50. **Strauch, M., V. Webb, G. Spiegelman, and J. A. Hoch.** 1990. The Spo0A protein of *Bacillus subtilis* is a repressor of the *abrB* gene. Proc. Natl. Acad. Sci. USA **87:**1801–1805.
51. **Tomsett, L., J. E. Craig, and A. L. Sonenshein.** 1997. Unpublished data.
52. **Towbin, H., T. Staehelin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76:**4350–4354.
53. **York, K., T. J. Kenney, S. Satola, C. P. Moran, Jr., H. Poth, and P. Youngman.** 1992. Spo0A controls the σ^A-dependent activation of the *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. J. Bacteriol. **174:**2648–2658.
54. **Yousten, A. A., and R. S. Hanson.** 1972. Sporulation of tricarboxylic acid cycle mutants of *Bacillus subtilis*. J. Bacteriol. **109:**886–894.
55. **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.