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Identification of Two Distinct *Bacillus subtilis* Citrate Synthase Genes

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Two distinct *Bacillus subtilis* genes (*citA* and *citZ*) were found to encode citrate synthase isozymes that catalyze the first step of the Krebs cycle. The *citA* gene was cloned by genetic complementation of an *Escherichia coli* citrate synthase mutant strain (W620) and was in a monocistronic transcriptional unit. A divergently transcribed gene, *citR*, could encode a protein with strong similarity to the bacterial LysR family of regulatory proteins. A null mutation in *citA* had little effect on citrate synthase enzyme activity or sporulation. The residual citrate synthase activity was purified from a *citA* null mutant strain, and the partial amino acid sequence for the purified protein (CitZ) was determined. The *citZ* gene was cloned from *B. subtilis* chromosomal DNA by using a PCR-generated probe synthesized with oligonucleotide primers derived from the partial amino acid sequence of purified CitZ. The *citZ* gene proved to be the first gene in a tricistronic cluster that also included *citC* (coding for isocitrate dehydrogenase) and *citH* (coding for malate dehydrogenase). A mutation in *citZ* caused a substantial loss of citrate synthase enzyme activity, glutamate auxotrophy, and a defect in sporulation.

The Krebs cycle provides important biosynthetic precursors (such as 2-ketoglutarate, succinyl coenzyme A [succinyl-CoA], and oxaloacetate) and plays a key role in energy production when glycolysis is unable to fulfill the cell's needs. Citrate synthase (acetyl-CoA + oxaloacetate→citrate + CoA) catalyzes the first, rate-limiting step of the Krebs cycle (13). The key metabolic position of citrate synthase has led to extensive investigation of its structure, enzymatic mechanism, and regulation in a variety of organisms, ranging from eubacteria to mammals (3, 17, 22, 25–27, 30, 39, 40). Generally, citrate synthases from gram-positive bacteria and from eukaryotes have relatively low subunit molecular weights, form dimers, and are inhibited by ATP. Citrate synthases from gram-negative bacteria, on the other hand, have relatively high subunit molecular weights, form hexamers, and are inhibited by 2-ketoglutarate and NADH. Inhibition of the initial enzyme of the Krebs cycle by ATP or NADH (reflecting energy status) constitutes an end-product feedback control process governing the central pathway of oxidative metabolism.

Upon nutritional starvation, *Bacillus subtilis*, a gram-positive bacterium, enters stationary phase and initiates a series of morphological changes that lead to formation and release of endospores (36). Spores are metabolically dormant and resistant to many chemical and physical agents that would kill normal, growing cells. Mutations that impair the function of the Krebs cycle in *B. subtilis* result in sporulation deficiency and the requirement for certain metabolic intermediates of the Krebs cycle (1, 9). It is unclear at present whether the sporulation defect is due to insufficient production of energy and biosynthetic precursors or to a failure to create a biochemical signal necessary for sporulation or both (41).

Regulation of synthesis of Krebs cycle enzymes in *B. subtilis* is complex (13). Although activities of all Krebs cycle enzymes are induced as cells enter stationary phase in a nutrient broth medium and are repressed by rapidly utilizable carbon sources (such as glucose), genes coding for the various steps in the Krebs cycle are regulated differently and respond differently to the presence of glucose (for reviews, see references 13 and 36).

Expression of citrate synthase and aconitase (the *citB* gene product) of *B. subtilis* appears to be coordinately regulated, at least at the level of enzyme activity (8). Interestingly, *citB* is not expressed in a strain that lacks citrate synthase enzyme activity, unless citrate is added to the medium (5). These findings led to the hypothesis that citrate, the product of citrate synthase, acts as an inducer of *citB* expression and that the activity of citrate synthase might influence later steps in the Krebs cycle (5, 10).

The nature of the *B. subtilis* gene coding for citrate synthase has not previously been identified. Since the first enzymatic step in the Krebs cycle is usually rate limiting, we sought to isolate and characterize this gene in order to probe both its regulation and its proposed functional role in metabolism and sporulation. Here we report that the *B. subtilis* genome has two distinct genes that encode citrate synthase enzymes.

MATERIALS AND METHODS

Bacterial strains and plasmids. All bacterial strains and relevant plasmids used in this study are listed in Table 1.

DNA manipulations, transformation, and culture media. Methods for restriction enzyme digestion, mung bean nuclease treatment, DNA ligation, and Southern hybridizations were as described by Maniatis et al. (18). Deletions in, insertions into, and integrations into *B. subtilis* chromosomal DNA were confirmed by Southern hybridizations. Radiolabelled DNA probes were generated by using the Ambion DECAprime DNA labelling kit (Ambion, Inc.) as described by the manufacturer. Preparation of electroporation-competent *Escherichia coli* cells and transformation by electroporation with a Bio-Rad Gene Pulser apparatus (Bio-Rad Laboratories) were as described by Dower et al. (6). When appropriate, the growth medium for *E. coli* contained 100 μg of ampicillin per ml or 200 μg of spectinomycin per ml. Preparation and transforma-

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TABLE 1. Bacterial strains and plasmids 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} *lacZΔM15*]	Laboratory stock
DH5α	F⁻ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* λ⁻ *thi-1 gyrA relA1*	Laboratory stock
W620	*thi-1 pyrD36 gltA6 galK30 rpsL129*	J. Guest
CJ236	*dut ung thi relA spoT1*(pCJ105)	J. Driscoll
B. subtilis		
1A32	*trpC2 citC6*	BGSC[a]
JH642	*trpC2 pheA1*	J. Hoch
MB186	SPβ^s *trpC2*	20
MH5143	*trpC2 pheA1 citH::tet*	M. Hulett
SJB9	Δ*citA::neo*	pCS11→SMY
SJB33	*trpC2 pheA1* Δ*citA::neo*	pCS11→JH642
SJB60	*citC::cat*	pCS51→SMY
SJB61	*trpC2 pheA1 citC::cat*	pCS51→JH642
SJB62	*trpC2 pheA1 citC::cat citC*⁺	pCS60→JH642
SJB66	*pheA1* Δ*citZ471*	pCS52 + SMY DNA→JH642
SJB67	*pheA1* Δ*citA::neo* Δ*citZ471*	pCS52 + SMY DNA→SJB33
SJB68	*pheA1* Δ*citA::neo* Δ*citZ47* SPβ*citZ erm*	SPβ*citZ*→SJB67
SJB191	*trpC2 pheA1 citZC::cat citZC*⁺	pCS71→JH642
SMY	Prototroph	Laboratory stock
ZB307A	SPβ*c2* Δ2::Tn*917*::pSK10Δ6 *erm*	43
Plasmids		
pAF23	*bla cat lacZ*	11
pBS⁻	*bla*	Stratagene, Inc.
pSK±	*bla*	Stratagene, Inc.
pJL74	*bla spc*	J. LeDeaux
pJPM1	*bla cat*	20
pCS4	*bla citA*⁺	This work
pCS4D	*bla citA**[b]	This work
pCS11	*bla* Δ*citA::neo*	This work
pCS24	*bla citA*⁺ *citR*⁺	This work
pCS34	*bla citZ*⁺ *citC′*	This work
pCS44	*bla cat citZ*⁺ *citC′*	This work
pCS51	*bla cat 'citC′*	This work
pCS52	*bla* Δ*citZ471 citC′*	This work
pCS60	*bla cat 'citC citH*⁺	This work
pCS71	*bla cat 'citZ citC′*	This work
pCS74	*bla citZ::spc citC′*	This work

[a] BGSC, *Bacillus* Genetic Stock Center, Columbus, Ohio.
[b] *citA** is a mutant form of *citA* in which the codon for glutamate at residue 307 has been replaced by an aspartate codon.

tion of competent *B. subtilis* cells have been described previously (10). Selection for drug resistance was on DS medium (10) plates containing chloramphenicol (2.5 μg/ml), neomycin (5 μg/ml), spectinomycin (100 μg/ml), or erythromycin (0.5 μg/ml) and lincomycin (12.5 μg/ml). TSS and LB media were as described previously (10).

Complementation of *E. coli* citrate synthase mutant strain W620. A library of *B. subtilis* chromosomal DNA partially digested with *Sau*3AI and cloned in plasmid pBR327 was constructed by A. Fouet in our laboratory. Another library of *B. subtilis* chromosomal DNA cloned in pJPM1 was constructed by J. P. Mueller (34). Both libraries were used to transform *E. coli* W620. Transformants were plated on glucose-ammonia minimal medium plates supplemented with vitamin B_1 (50 μg/ml), uracil (150 μg/ml), and ampicillin (100 μg/ml) to select colonies prototrophic for glutamate. Plasmid DNA from purified glutamate prototrophs was used to retransform strain W620 to verify complementation. Two plasmids that restored the glutamate prototrophy were named pCS0 (isolated from the pBR327 library) and pCS24 (isolated from the pJPM1 library) (Fig. 1). The entire DNA insert carried in plasmid pCS0 was subcloned into pSK⁺, creating plasmid pCS4.

Assays of citrate synthase, isocitrate dehydrogenase (ICDH), and aconitase enzyme activities. *B. subtilis* cells for enzyme activity assays were grown in 100 ml of DS medium to 30 min after the end of exponential growth phase. *E. coli* cells were grown in 100 ml of L broth for 15 h at 37°C. In both cases, cells were harvested and washed with a buffer containing 20 mM Tris (pH 8.0), 1 mM EDTA, and 20% glycerol, frozen immediately on dry ice, and stored at −20°C for later use. Cell pellets were resuspended in 2 ml of the same buffer prior to sonication (40 s; Branson Sonifier Cell Disrupter 200). Cell debris was eliminated by centrifugation (15 min at 12,000 × *g*, 4°C), and supernatant fluids were used for enzyme activity assays.

Enzyme activities of citrate synthase, ICDH, and aconitase were assayed spectrophotometrically at 25°C according to the procedures described by Fortnagel and Freese (9) and Hanson and Cox (12). Units of citrate synthase, aconitase, and ICDH were expressed as micromoles of CoA produced per minute per milligram of protein, nanomoles of *cis*-aconitate produced

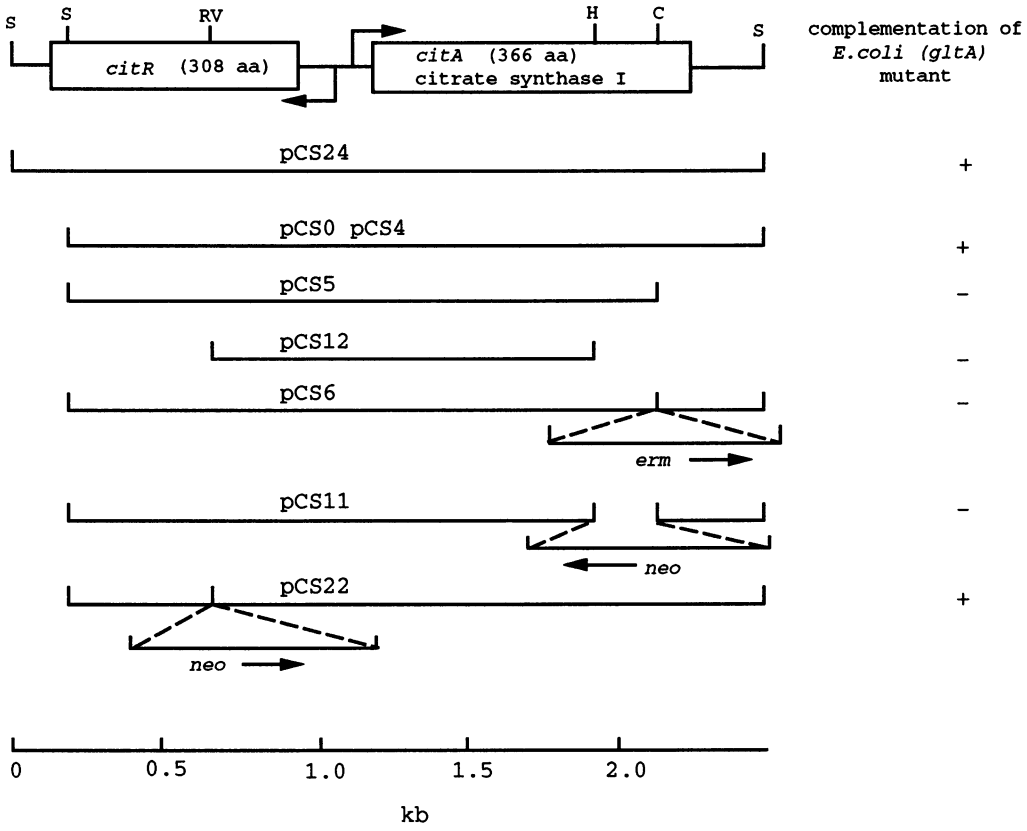

FIG. 1. Physical map of the *citR-citA* region. Deletion and insertion mutations of *citA* and their effects on complementation of an *E. coli gltA* mutant are shown. Orientations of *citA* and *citR* ORFs are indicated by arrows. Restriction sites: S, *Sau*3AI; RV, *Eco*RV; H, *Hin*dIII; C, *Cla*I. Not all *Sau*3AI sites are shown. aa, amino acids.

per minute per milligram of protein, and nanomoles of NADPH produced per minute per milligram of protein, respectively. Protein concentrations were determined by the Bio-Rad Coomassie blue assay (10).

Deletion-insertion mutations in *citA* and *citR*. Plasmid pCS5 (Fig. 1) was constructed by digesting plasmid pCS0 with *Cla*I and religating. This removes a 730-bp *Cla*I fragment extending from the *Cla*I site in *citA* to the *Cla*I site in the vector and results in the loss of 49 amino acids of CitA at the C-terminal end. Plasmid pCS6 was constructed by inserting the *Cla*I-digested erythromycin resistance (*erm*) cassette from pJPM9 (20) into the *Cla*I site of pCS4; the orientation of the *erm* gene is such that transcription is in the same orientation as that of the *citA* gene. Plasmid pCS12 was constructed by cloning the *Hin*dIII-*Eco*RV insert DNA from plasmid pCS4 into the *Hin*dIII and *Eco*RV sites of pSK$^+$, which results in the loss of 103 amino acids of CitA at the C-terminal end. Plasmid pCS11 was constructed in three steps. First, to create pCS3, a neomycin resistance (*neo*) cassette was excised by *Sma*I digestion from plasmid pBEST501 (15) and ligated to pCS0 that had been cleaved with *Hin*dIII and treated with Klenow fragment of DNA polymerase I to form blunt ends. *Hin*dIII digestion of pCS0 creates a large fragment, containing most of the vector and the N-terminal coding region of *citA*, and a small fragment, which was discarded. Second, a fragment extending from the *Hin*dIII site in *citA* to the *Sac*I site in the vector component of pCS4 was cloned into the *Hin*dIII and *Sac*I sites of pSK$^+$ to create plasmid pCS9. Third, the *Eco*RV-*Cla*I insert DNA from plasmid pCS3 was cloned into the large *Hin*cII-*Cla*I fragment of pCS9 to create pCS11. Plasmid pCS11 contains a *neo* insertion after codon 263 of *citA* and a deletion of codons 264 to 317. Plasmid pCS22 was constructed by inserting the *Sma*I fragment carrying the *neo* cassette from pBEST501 into the *Eco*RV site of plasmid pCS4; the orientation of the *neo* gene is such that transcription is in the orientation opposite to that of the *citR* gene.

Purification of CitZ. The residual citrate synthase enzyme activity was purified from a *citA* null mutant strain, SJB9, according to the method described by Robinson et al. (26) with minor modifications. The purified protein was transferred to Immobilon-P for N-terminal amino acid sequencing. The purified protein was also partially digested by trypsin, and the tryptic peptides were separated by high-pressure liquid chromatography (HPLC) for amino acid sequencing. Protein sequencing and separation by HPLC of tryptic peptides were carried out by M. Berne in the Tufts University Protein and Nucleic Acid Analysis Unit.

Cloning of *citZ*. PCR primers were designed against the N-terminal and internal amino acid sequences according to the codon usage of *B. subtilis* (33) and synthesized by the Tufts University Protein and Nucleic Acid Analysis Unit. A 26-base degenerate oligonucleotide [5′-GAC(T/A)GCTAC(T/A)(C/A)G(T/C/A)GG(T/C/A)(T/C)T(A/T)GA(A/G)TGCG-3′] was designed against the N-terminal protein sequence, and a 30-base "guessmer" oligonucleotide (5′-GTTTTCAACTTCA ACAATTTCTGTAAGCAT-3′) was designed against an internal protein sequence and was based on codon preferences for *B. subtilis* (33). PCRs were performed in 50-μl mixtures,

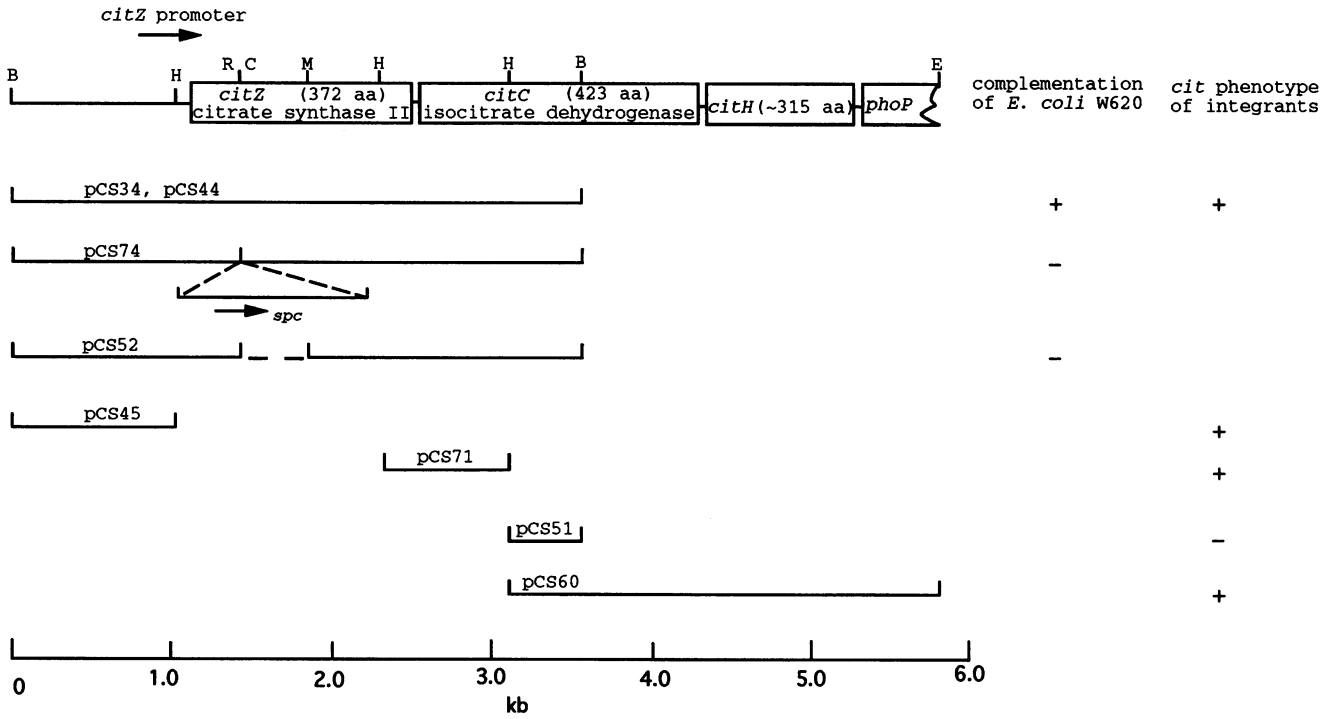

FIG. 2. Physical map of the *citZ* region. The direction of transcription of the operon is indicated by an arrow. The locations of a *spc* insertion mutation and an in-frame deletion within *citZ* are indicated. The ability of certain plasmids to complement the *gltA* mutation of *E. coli* W620 and the effect of the integration of certain plasmids into the *B. subtilis* chromosome are also shown. Restriction sites: B, *Bgl*II; H, *Hin*dIII; C, *Cla*I; M, *Mlu*I; E, *Eco*RI; R, *Eco*RV. aa, amino acids.

covered with 100 μl of light mineral oil, in a thermal cycler (MJ Research, Inc.). The reaction mixture included 10 pmol of each primer, 2 μg of *B. subtilis* SMY chromosomal DNA, 2.5 U of *Taq* DNA polymerase (Bethesda Research Laboratories), 20 mM Tris (pH 8.0), 50 mM KCl, 1 mM $MgCl_2$, and 100 nM each dATP, dCTP, dGTP, and TTP. The reaction mixture was incubated for 3 min at 95°C, amplified for 30 cycles (15 s at 94°C, 30 s at 45°C, and 30 s at 72°C), and then extended for 5 min at 72°C.

The predicted amplified product of 800 bp was obtained. The same band was obtained, however, even when only the internal primer was used and even when very stringent annealing conditions were used. The degenerate oligonucleotide corresponding to the N-terminal amino acid sequence did not seem to contribute to the PCR product. Despite its anomalous appearance, the 800-bp amplified fragment was cloned and sequenced. It proved to encode a peptide sequence with about 40% identity to the N-terminal 260 amino acids of several bacterial citrate synthase proteins. (Subsequent DNA sequence analysis showed that the 3′ end of the guessmer oligonucleotide has a fortuitous 9-nucleotide perfect match with a DNA sequence near the 5′ end of the *citZ* gene. The degenerate oligonucleotide had insufficient homology to the actual N-terminal coding sequence to anneal under the conditions used.)

When the cloned 800-bp PCR fragment was radioactively labelled and used to probe a Southern blot of *Bgl*II-digested *B. subtilis* chromosomal DNA, a single hybridizing band of about 4 kb was detected. A mixture of chromosomal *Bgl*II fragments of about 4 kb was excised from an agarose gel, ligated to *Bam*HI-digested pBS^-, and introduced into the *E. coli gltA* mutant strain W620 to test for complementation. Ten complementing clones each had a 3.8-kb *Bgl*II fragment inserted in the vector. These plasmids (e.g., pCS34) were identical as determined by restriction enzyme analysis.

Spectinomycin cassette insertion mutation of *citZ*. A 1.1-kb *Eco*RV-*Bam*HI fragment containing the spectinomycin adenyltransferase gene (*spc*) and its transcriptional terminator was excised from plasmid pJL74, gel purified, treated with the Klenow fragment of DNA polymerase I to fill in the protruding *Bam*HI 5′ end, and ligated with pCS34 (Fig. 2) that had been cleaved with *Eco*RV. The ligation mixture was used to transform *E. coli* DH5α cells. Plasmid pCS74 was recovered from one such transformant, and its structure was verified by restriction enzyme analysis. The orientation of the *spc* gene in pCS74 is such that transcription is in the same direction as that of the *citZ* gene.

Production of SPβ*citZ* transducing phage and complementation of *citZ* mutants. An SPβ specialized transducing phage carrying the entire *citZ* gene was constructed by methods described previously (20). Briefly, pCS44, a derivative of the integration plasmid pJPM1 (20) that carries the entire 3.8-kb DNA insert from plasmid pCS34 (Fig. 2), was used to transform competent cells of strain ZB307A to chloramphenicol resistance (Cam^r). The Cam^r transformants were pooled, and an SPβ transducing lysate was generated and used to infect exponentially growing cells of SPβ-sensitive strain MB186. A Cam^r lysogen of MB186 was used as a source of SPβ*citZ* specialized transducing phage.

SPβ*citZ* phage was used to infect *B. subtilis* SJB66 and SJB67, with selection for chloramphenicol resistance. Cam^r transductants were streaked onto TSS glucose-ammonia minimal medium plates to test for glutamate prototrophy.

Chromosomal walking to clone *citC*. Plasmid pCS51, a

derivative of pJPM1 that carries the 400-bp *Hin*dIII-*Bgl*II DNA fragment downstream of *citZ* (Fig. 2), was integrated into the chromosome of *B. subtilis* SMY by selecting for Cam^r. Chromosomal DNA (2 μg) of the resulting strain, SJB60, was digested with *Eco*RI, ligated under dilute conditions (7 ng/μl), and introduced into *E. coli* DH5α by electroporation with selection for Amp^r. Plasmid DNA was isolated from *E. coli* transformants for restriction analysis. A plasmid carrying a 2.3-kb *Hin*dIII-*Eco*RI DNA fragment (pCS60) was chosen for further studies.

Plasmid pCS71 has an 872-bp *Hin*dIII fragment that includes the 3′ end of *citZ* and the 5′ end of *citC* inserted in pJPM1.

***citZ* in-frame deletion.** Plasmid pCS34 was digested with *Eco*RV and *Mlu*I (blunt ended by mung bean nuclease treatment) to remove 471 bp of DNA of the *citZ* coding region and religated (Fig. 2). After DNA sequencing confirmed the status of the in-frame deletion of *citZ* in plasmid DNA (named pCS52), the deletion was transferred to the *B. subtilis* chromosome by transforming strains SJB33 and JH642 with 50 ng of SMY chromosomal DNA and saturating amounts of plasmid pCS52. Trp$^+$ transformants were selected on TSS glucose-glutamate-phenylalanine minimal medium plates and screened for the acquisition of a Glt$^-$ phenotype as evidenced by poor growth on TSS glucose-ammonia-phenylalanine plates. The deletion in the *B. subtilis* chromosome was confirmed by PCR and Southern hybridization assays. SPβ*citZ* transducing phage was used to infect the resulting *citZ* in-frame deletion strains of *B. subtilis* by selecting for Cam^r on DS medium. Cam^r transductants were tested for glutamate prototrophy on TSS glucose-ammonia-phenylalanine minimal medium plates.

Site-directed mutagenesis of the *citA* gene. A 23-base oligonucleotide, OSJ14 (CACAAATGTTGATTTTTACGCAG), corresponding to nucleotides 2039 to 2061 of the nontemplate strand of the *citA* gene was synthesized. OSJ14 specifies aspartate (GAT) instead of glutamate (GAA) at codon 307 of CitA. Single-stranded DNA (template strand) was obtained for plasmid pCS9 (a pSK$^+$ derivative that carries a 700-bp fragment encoding the C-terminal portion of CitA), using *E. coli* CJ236 as described previously (42). OSJ14 was phosphorylated at the 5′ end by T4 polynucleotide kinase (18); annealed with single-stranded pCS9 template at 70°C for 5 min; cooled gradually to room temperature in a buffer containing 40 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 50 mM NaCl; extended with T7 DNA polymerase at 37°C for 90 min in a buffer containing 0.5 mM each dGTP, dATP, TTP, and dCTP, 1 mM ATP, 50 mM Tris (pH 8.0), 7 mM $MgCl_2$, and 10 mM dithiothreitol; and finally ligated with T4 DNA ligase. The ligation mixture was used to transform *E. coli* JM107 to Amp^r, and plasmid DNA was recovered from 10 transformants for DNA sequencing analysis. A plasmid that had the desired substitution of GAT (Asp) for GAA (Glu) but that did not contain any other changes within the 700-bp insert DNA was named pCS9D. The *Hin*dIII-*Sac*I fragment of pCS9D was subcloned into the *Hin*dIII and *Sac*I sites of plasmid pCS4 (Fig. 1), and the resulting plasmid (named pCS4D) was shown by DNA sequencing to contain the desired substitution.

DNA sequence analysis. *B. subtilis* chromosomal inserts (cloned in pBS$^-$ or pSK$^\pm$) were sequenced by the dideoxy chain termination method (29) with modified T7 DNA polymerase (Sequenase, version 2.0; U.S. Biochemical Corp.) or *Taq* DNA polymerase (Cetus) and vector- or insert DNA-specific oligonucleotides. Plasmid DNA to be sequenced was purified by CsCl gradient centrifugation or by the alkaline lysis protocol of Maniatis et al. (18). Single-stranded DNA templates were prepared with helper phage R408 as described previously (28). DNA sequence analysis was performed by using the package of programs provided by the University of Wisconsin Genetics Computer Group (4).

TABLE 2. Citrate synthase enzyme activities of *E. coli* strains

Strain	Sp act[a]
DH5α	0.261
W620(pSK$^-$)	0.002
W620(pCS4)	0.106
W620(pCS4D)	0.484
W620(pCS34)	0.515

[a] See text for units.

Nucleotide sequence accession numbers. The sequence information reported in this study has been deposited in the GenBank database under accession numbers U05256 for the *citR-citA* locus and U05257 for the *citZCH* locus.

RESULTS

Cloning of *citA* by complementation of an *E. coli* citrate synthase mutant. We cloned the *B. subtilis citA* gene by complementation of an *E. coli* citrate synthase (*gltA*) mutant strain, W620, using plasmid DNA libraries of *B. subtilis* (SMY) chromosomal DNA (see Materials and Methods). Several plasmids (pCS0 and pCS24) were able to restore strain W620 to glutamate prototrophy; they carried overlapping inserts of *B. subtilis* DNA (Fig. 1). The citrate synthase enzyme activity of strain W620(pCS4) was approximately 80-fold higher than that of strain W620(pSK$^-$) (Table 2). [The citrate synthase specific activity of strain W620(pCS4) was about 40% of that of the (nonisogenic) *gltA*$^+$ strain DH5α.] *E. coli* W620(pCS4) grew as well as a wild-type *E. coli* strain in glucose-ammonia minimal medium (data not shown). To verify that the DNA insert carried in plasmid pCS4 was derived from the *B. subtilis* chromosome, we showed that pCS4 hybridized to Southern blots of chromosomal DNAs of *B. subtilis* SMY and JH642.

Deletion and insertion mutagenesis of *citA*. As shown in Fig. 1, certain deletion and insertion mutations within the 2.2-kb DNA carried by pCS4 abolished complementation of the *E. coli gltA* mutant. Citrate synthase activity was reduced to the background level in strain W620(pCS11), which carries a deletion-insertion mutation of the *citA* gene (data not shown). These results strongly suggest that pCS4 carries a functional copy of the *citA* gene of *B. subtilis*.

Similarities of *citA* to citrate synthase genes and of *citR* to LysR family genes. The sequence of the insert in pCS24 was determined on both strands and revealed two potential open reading frames (ORFs), transcribed in opposite orientations and termed *citA* and *citR* (Fig. 3). *citA* and *citR* encode potential proteins of 366 amino acids (CitA) and 308 amino acids (CitR). Mutations in the *citA* ORF abolished complementation of *E. coli* W620; a mutation in *citR* did not (Fig. 1).

The putative product of *citA* (molecular weight, 40,937) was similar in amino acid sequence to citrate synthases from bacteria and mammals (3, 22, 27, 30, 40) (Table 3). This suggested that we had indeed cloned a gene encoding citrate synthase. The amino acid residues that are thought to be the substrate-binding site and catalytic site are underlined in Fig. 3 and in most cases match the consensus residues (25, 39). The putative product of the upstream ORF, CitR, has similarity to the LysR family of bacterial regulatory proteins, including the *E. coli* positive regulatory protein OxyR (37) (20.4% identity) and the *Acinetobacter calcoaceticus* repressor protein CatM (21) (23.1% identity). The putative CitR helix-turn-helix DNA-binding motif characteristic of LysR family proteins is

FIG. 3. DNA sequence of the nontemplate strands of *citA* and *citR*. *citA* and *citR* are transcribed in opposite orientations, with 92 nucleotides between the predicted translational initiation codons. Only the nontemplate strands are shown, except for the intergenic region, for which both strands are presented. CitA residues at the positions of substrate-binding sites and catalytic sites in other citrate synthase enzymes are underlined. CitR residues likely to form a helix-turn-helix motif are also underlined. Putative factor-independent transcriptional terminators are indicated by >.

underlined in Fig. 3. CitR is a negative regulator of *citA* transcription (16).

Transformation of *B. subtilis* with a disrupted *citA* gene. The insertion mutation in *citA* carried on plasmid pCS11 (Fig. 1) was introduced into the chromosomes of *B. subtilis* SMY and JH642 by double crossover recombination. Unexpectedly, the resulting strains, SJB9 and SJB33, respectively, did not appear to have any defect in citrate synthase enzyme activity or sporulation frequency and were not glutamate auxotrophs. Either *citA* is not a citrate synthase gene or *B. subtilis* SMY and JH642 each have two functional genes for citrate synthase. Since a *citA-lacZ* transcriptional fusion was expressed and since *citA* mRNA could be detected in an RNase T_2 protection experiment (16), we could rule out the possibility that *citA* is an unexpressed pseudogene.

Cloning and sequencing of *citZ*. The residual citrate synthase enzyme activity was purified from a *citA* null mutant strain (SJB9) as described in Materials and Methods. The purified protein (called CitZ) had an apparent mass of approximately 41 kDa, on the basis of mobility in sodium dodecyl sulfate-

TABLE 3. Comparison of citrate synthase sequences from *B. subtilis* and other organisms

B. subtilis citrate synthase type	Identity (%) to citrate synthase from[a]:							
	E. coli	*Rickettsia prowazekii*	*Mycobacterium smegmatis*	*Bacillus coagulans*	*S. cerevisiae*		*B. subtilis*	
					Cit-I	Cit-II	CitA	CitZ
CitA	30	33	39	37	20	27		42
CitZ	35	35	43	42	27	26	42	

[a] References: 3, 21, 26, 29, and 39. Sequences were compared by using the Bestfit program (4).

polyacrylamide gel electrophoresis, which was similar to the size of the CitA protein predicted from its DNA sequence. Purified CitZ protein appeared to form dimers in native polyacrylamide protein gels and had a K_m for oxaloacetate of 15 μM and a K_m for acetyl-CoA of 22 μM. These properties of the purified CitZ protein were consistent with those of the citrate synthase enzymes from gram-positive bacteria and eukaryotes (30, 39). The sequences of the N-terminal amino acids (MTATRGLEGVVATTSSVSSII) and of an internal tryptic peptide (MLTEIGEVEN) of the purified CitZ protein were similar, but not identical, to corresponding regions of the deduced amino acid sequence of *citA*. The internal amino acid sequence of the purified protein was also homologous to those of several other citrate synthase proteins.

PCR primers were used to amplify a portion of the *citZ* gene from the chromosomal DNA of *B. subtilis* SMY. The cloned PCR product (~800 bp) was then used as a probe to identify and clone a 3.8-kb *Bgl*II fragment of the chromosome (see Materials and Methods). Ten identical clones were obtained, each capable of restoring *E. coli* strain W620 to glutamate prototrophy. The insert in one of the plasmids (pCS34 [Fig. 2]) was sequenced on both strands (Fig. 4). The N-terminal and internal amino acid sequences predicted by the DNA sequence of the *citZ* gene were identical to those determined from the purified CitZ protein. We conclude that the *citZ* gene, contained in plasmid pCS34, codes for the citrate synthase enzyme that is active in a *citA* mutant strain of *B. subtilis*. When present in *E. coli* W620, pCS34 caused a 370-fold increase in citrate synthase enzyme activity (Table 2), yielding a specific activity twofold higher than that of strain DH5α. A spectinomycin adenyltransferase gene cassette (*spc*) from plasmid pJL74 was introduced into the *citZ* coding region, creating plasmid pCS74 (see Materials and Methods). This insertion mutation in *citZ* abolished its ability to complement *E. coli* citrate synthase mutant W620 (Fig. 2).

Similarity of *citZ* to other citrate synthase genes. The putative amino acid sequence of *citZ* has 26 to 43% identity with those of known citrate synthase proteins (3, 22, 27, 30, 40) (Table 3). Residues thought to be involved in substrate binding and catalysis are indicated in Fig. 4; they were highly conserved in CitZ (25, 39). *citA* and *citZ* have 42% identity at the amino acid sequence level and 59% identity at the DNA sequence level. We conclude that there are two distinct homologous citrate synthase genes in *B. subtilis*.

Mapping of *citA* and *citZ*. Using an ordered library of *B. subtilis* DNA in yeast artificial chromosomes and probes made from plasmids pCS4 (Fig. 1) and pCS34 (Fig. 2), P. Serror mapped *citA* and *citZ* to approximately 90° and 250°, respectively, on the *B. subtilis* chromosome (32). We used two-factor transformation crosses to show that *citA* is 2% linked to the *sspB* gene, which encodes a small, acid-soluble protein (19), and that *citZ* is 11% linked to *phoP*, a gene encoding a regulator of alkaline phosphatase (31).

***citZ* in-frame deletion.** To test the specific role of *citZ* in growth and sporulation, we constructed a *citZ* in-frame deletion (see Materials and Methods). Plasmid pCS52, with a deletion of 157 amino acids within the CitZ protein, was unable to complement *E. coli* W620 (Fig. 2). The in-frame deletion was introduced into *B. subtilis* JH642 and SJB33 (Δ*citA*::*neo*), and the two resulting strains, SJB66 and SJB67, were tested for glutamate auxotrophy. Strain SJB66 (Δ*citZ471*) showed a slow-growth phenotype in glucose-ammonia minimal medium, while strain SJB67 (Δ*citA*::*neo* Δ*citZ471*) had an absolute requirement for glutamate. Growth of both strains was restored to the wild-type rate in the presence of glutamate. Both strains also had defects in citrate synthase enzyme activity and in sporulation (see below). All of these deficiencies of strains SJB66 and SJB67 were fully complemented by SPβ*citZ*. We conclude that the *citZ* in-frame deletion disrupts the gene for the cell's major citrate synthase and is not strongly polar on the expression of downstream genes.

Limits of the *citZ* transcription unit. To define the extent of upstream DNA sequence necessary for *citZ* expression, plasmids pCS44 and pCS45 (pJPM1 derivatives) were integrated into the chromosome of *B. subtilis* JH642 (Fig. 2). The resulting strains were glutamate prototrophs and sporulated well. The inserts in these plasmids must contain the 5′ end of the *citZ* transcription unit.

Plasmid pCS51, which carries a 400-bp DNA fragment downstream of *citZ* (Fig. 2), was also integrated into the chromosome of *B. subtilis* SMY and JH642. The resulting strains, SJB60 and SJB61, respectively, were sporulation defective and auxotrophic for glutamate. Therefore, at least one gene downstream of *citZ* is needed for sporulation and synthesis of glutamate and might be in the same transcription unit with *citZ*. We sought to isolate and characterize that gene.

Cloning and sequencing of the ICDH (*citC*) and malate dehydrogenase (*citH*) genes. The DNA sequence immediately downstream of the *citZ* gene in plasmid pCS34 revealed an ORF with a predicted amino acid sequence with strong similarities to those of ICDHs. However, plasmid pCS34 did not appear to have the entire coding region for isocitrate dehydrogenase, on the basis of the glutamate auxotrophy of strain SJB60 and comparison of the predicted amino acid sequence of the potential ORF with those of known ICDHs.

We cloned the 3′ end of the apparent ICDH gene by chromosomal walking (see Materials and Methods). A plasmid (pCS60) that carries a 2.3-kb *Hin*dIII-*Eco*RI fragment that overlaps with the downstream end of the DNA carried in plasmid pCS34 (Fig. 2) was isolated and integrated into the *B. subtilis* chromosome. The resulting strain, SJB62, was a glutamate prototroph and sporulated well, indicating that plasmid pCS60 contains the 3′ end of a transcription unit. DNA sequencing of the insert in pCS60 revealed two potential ORFs. The ORF that appears to code for ICDH has 423 codons, and its putative product has 66.7 and 33.3% identities to the ICDHs of *E. coli* (38) and *Saccharomyces cerevisiae* (2), respectively. It is clear that this ORF corresponds to the *citC*

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                                  > >>>>>>>>>>>>          <<<<<<<<<<<< <
1     TAGCCATGCAGATCGTAAAACTGTTCACTTCATAAAAAAAACCCCTTAAAGCCGCTTTTTTTTATGCAAAAACCCGCAAATAAAGGCGTTTTTTCGGGGA
101   AATCACAGAATGGAAAAAAAAAATTATAGGTAAACATTTAACAAATGTCTGATTATTGTTTATAATGAGAATAGGCTTAAACTTAAATAAGCTTATAAGA
201   ATTTGTTATGTTCTTTCATAAGCAAAGGGTTTTAGTTCCAGCAGCCAGTAAAATGTAAGCATTTTCTTTTTGGGGGAGAGAAATACTTGCATCGTGCTTT
                                                                citZ --> M  T  A  T  R  G  L  E  G  V  V  A
301   TTAAGTAAGCCTCATGTTTTTACAACACTCTTAAAGGGGAAATTTATTGAAAAGGAGATGTTATATATGACAGCGACACGCGGTCTTGAAGGGGTTGTAG
        T  T  S  S  V  S  S  I  I  D  D  T  L  T  Y  V  G  Y  D  I  D  D  L  T  E  N  R  S  F  E  E  I  I
401   CAACAACATCATCTGTTAGTTCTATTATTGATGATACCCTTACATATGTGGGGTATGATATCGATGATTTGACAGAGAATCGAAGTTTTGAAGAAATCAT
        Y  L  L  W  H  L  R  L  P  N  K  K  E  L  E  E  L  K  Q  Q  L  A  K  E  A  A  V  P  Q  E  I  I  E
501   CTACTTGCTTTGGCATCTAAGGCTGCCAAACAAAAAAGAGCTTGAAGAGTTAAAGCAGCAGCTTGCAAAGGAAGCTGCCGTTCCGCAAGAAATTATTGAG
        H  F  K  S  Y  S  L  E  N  V  H  P  M  A  A  L  R  T  A  I  S  L  L  G  L  L  D  S  E  A  D  T  M  N
601   CACTTCAAATCTTATTCCCTCGAAAATGTTCATCCCATGGCTGCTCTTCGGACAGCAATTTCATTACTAGGCCTGCTTGACAGCGAGGCTGATACCATGA
        P  E  A  N  Y  R  K  A  I  R  L  Q  A  K  V  P  G  L  V  A  A  F  S  R  I  R  K  G  L  E  P  V  E
701   ACCCGGAAGCCAACTACAGAAAGGCGATCCGCCTTCAGGCAAAGGTGCCTGGCCTGGTTGCAGCGTTTTCCAGAATTCGCAAGGGTCTTGAGCCTGTTGA
        P  R  E  D  Y  G  I  A  E  N  F  L  Y  T  L  N  G  E  E  P  S  P  I  E  V  E  A  F  N  K  A  L  I
801   ACCGAGAGAAGATTACGGTATTGCGGAGAATTTTCTTTACACATTAAACGGTGAAGAGCCTTCACCAATTGAAGTTGAAGCCTTCAACAAAGCATTGATT
        L  H  A  D  H  E  L  N  A  S  T  F  T  A  R  V  C  V  A  T  L  S  D  I  Y  S  G  I  T  A  A  I  G  A
901   TTACATGCTGACCATGAGCTGAACGCGTCAACGTTCACAGCGAGGGTCTGCGTCGCAACACTTTCTGACATCTATTCAGGCATTACTGCAGCAATCGGCG
        L  K  G  P  L  H  G  G  A  N  E  G  V  M  K  M  L  T  E  I  G  E  V  E  N  A  E  P  Y  I  R  A  K
1001  CGCTGAAAGGGCCTCTTCACGGCGGAGCGAATGAAGGCGTTATGAAAATGCTGACAGAAATCGGTGAAGTGGAAAACGCTGAGCCGTACATCCGCGCTAA
        L  E  K  K  E  K  I  M  G  F  G  H  R  V  Y  K  H  G  D  P  R  A  N  D  L  K  E  M  S  K  R  L  T
1101  ACTTGAAAAGAAAGAAAAAATCATGGGATTCGGCCACCGTGTGTATAAGCATGGTGATCCGCGCGCGAACGATCTGAAAGAAATGAGCAAGCGCCTGACC
        N  L  T  G  E  S  K  W  Y  E  M  S  I  R  I  E  D  I  V  T  S  E  K  K  L  P  P  N  V  D  F  Y  S  A
1201  AACCTGACAGGCGAAAGCAAATGGTATGAGATGTCGATTCGTATAGAAGACATCGTCACATCAGAGAAAAAGCTTCCGCCTAACGTTGATTTCTATTCGG
        S  V  Y  H  S  L  G  I  D  H  D  L  F  T  P  I  E  A  V  R  R  M  S  G  W  L  A  H  I  L  E  Q  Y
1301  CTTCTGTATATCACAGCCTCGGTATTGACCATGATTTGTTTACACCGATTTTTGCTGTAAGACGAATGTCCGGCTGGCTCGCTCATATTCTTGAGCAGTA
        D  N  N  R  L  I  R  P  R  A  D  Y  T  G  P  D  K  Q  K  F  V  P  I  E  E  R  A  *
1401  TGACAACAACCGTCTGATCCGCCCGCGGGCAGATTACACAGGCCCTGACAAACAAAAATTCGTTCCGATTGAAGAAAGAGCCTAAAGAACCATTGGAGGC

1501  TGGCTTGCGTCAATGAGGTTAGCCTCTTGTTCAGACATCAAAATTGGGTTACACTTTAAATTGAATGTTAGGAAAAATCATTTTATTTTCTATATAAGAA
                                                  citC-->   M  A  Q  G  E  K  I  T  V  S  N  G  V  L  N  V  P  N
1601  AAATAAAATGCTTTCTAAATATGAATTACATACTGGGAGGTTTTTATTGTGGCACAAGGTGAAAAAATTACAGTCTCTAACGGAGTATTAAACGTACCAA
        N  P  I  I  P  F  I  E  G  D  G  T  G  P  D  I  W  N  A  A  S  K  V  L  E  A  A  V  E  K  A  Y  K
1701  ACAACCCGATTATCCCATTTATCGAAGGAGACGGAACCGGTCCTGATATTTGGAACGCGGCTTCGAAGGTTTTGGAAGCAGCAGTAGAAAAAGCATACAA
        G  E  K  K  I  T  W  K  E  V  Y  A  G  E  K  A  Y  N  K  T  G  E  W  L  P  A  E  T  L  D  V  I  R
1801  AGGCGAAAGAAAAATTACATGGAAAGAAGTTTACGCCGGAGAAAAGGCTTATAATAAAACAGGTGAGTGGCTCCCTGCTGAAACATTAGATGTGATCCGC
        E  Y  F  I  A  I  K  G  P  L  T  T  P  V  G  G  G  I  R  S  L  N  V  A  L  R  Q  E  L  D  L  F  V  C
1901  GAATATTTCATCGCGATTAAAGGCCCGTTAACGACACCTGTCGGCGGCGGTATCCGTTCTTTGAACGTAGCGCTCAGACAAGAGCTTGACCTATTCGTCT
        L  R  P  V  R  Y  F  T  G  V  P  S  P  V  K  R  P  E  D  T  D  M  V  I  F  R  E  N  T  E  D  I  Y
2001  GCTTAAGACCTGTAAGATACTTTACTGGAGTGCCGTCACCGGTAAAACGCCCTGAAGATACTGATATGGTCATCTTCCGTGAAAATACAGAAGATATTTA
        A  G  I  E  Y  A  K  G  S  E  E  V  Q  K  L  I  S  F  L  Q  N  E  L  N  V  N  K  I  R  F  P  E  T
2101  CGCAGGCATCGAGTATGCAAAAGGCTCTGAAGAAGTGCAAAAGCTTATCAGCTTCCTTCAAAATGAGTTAAACGTCAATAAAATCCGTTTCCCTGAGACT
        S  G  I  G  I  K  P  V  S  E  E  G  T  S  R  L  V  R  A  A  I  D  Y  A  I  E  H  G  R  K  S  V  T  L
2201  CAGGTATCGGCATTAAGCCTGTTTCAGAAGAAGGAACAAGCCGCTTGGTCAGAGCTGCCATTGATTATGCGATCGAGCAATGGCCGCAAATCTGTAACAC
        V  H  K  G  N  I  M  K  F  T  E  G  A  F  K  N  W  G  Y  E  L  A  E  K  E  Y  G  D  K  V  F  T  W
2301  TTGTTCACAAAGGAAACATCATGAAGTTCACAGAAGGCGCCTTCAAAAACTGGGGCTATGAACTTGCTGAAAAAGAATACGGAGATAAAGTCTTCACATG
        A  Q  Y  D  R  I  A  E  E  Q  G  K  D  A  A  N  K  A  Q  S  E  A  E  A  A  G  K  I  I  I  K  D  S
2401  GGCTCAATATGACCGCATTGCTGAAGAACAAGGAAAAGACGCTGCCAACAAAGCGCAAAGCGAAGCGGAAGCAGCAGGAAAAATCATTATCAAAGACAGC
        I  A  D  I  F  L  Q  Q  I  L  T  R  P  N  E  F  D  V  V  A  T  M  N  L  N  G  D  Y  I  S  D  A  L  A
2501  ATTGCTGACATTTTCCTTCAGCAGATCTTAACGCGTCCAAACGAGTTTGATGTCGTTGCGACAATGAACTTGAACGGAGATTACATTTCTGATGCTCTTG
        A  Q  V  G  G  I  G  I  A  P  G  A  N  I  N  Y  E  T  G  H  A  I  F  E  A  T  H  G  T  A  P  K  Y
2601  CTGCGCAAGTCGGCGGAATCGGCATTGCTCCTGGAGCGAACATCAACTACGAAACAGGACATGCGATTTTCGAGGCGACGCACGGAACGGCTCCTAAATA
        A  G  L  D  K  V  N  P  S  S  V  I  L  S  G  V  L  L  L  E  H  L  G  W  N  E  A  A  D  L  V  I  K
2701  TGCAGGCCTTGATAAAGTAAACCCATCTTCAGTTATTCTTTCAGGCGTTCTGCTTCTTGAGCATTTAGGATGGAACGAAGCGGCTGATTTGGTTATCAAA
        S  M  E  K  T  I  A  S  K  V  V  T  Y  D  F  A  R  L  M  D  G  A  T  E  V  K  C  S  E  F  G  E  E  L
2801  TCTATGGAAAAAACAATCGCTTCTAAAGTCGTAACTTACGATTTTGCCAGATTAATGGACGGGGCGACTGAAGTGAAATGTTCAGAGTTCGGAGAAGAAC
        I  K  N  M  D  *
2901  TGATCAAAAACATGGACTAAGCAAGGAAAAGCCTAAACTAGCCATAAGG
```

FIG. 4. DNA sequence of the nontemplate strand of *citZ* and *citC*. *citZ* and *citC* are transcribed in the same direction. A putative factor-independent transcriptional terminator upstream of *citZ* is indicated by >. CitZ residues at the positions of substrate-binding sites and catalytic sites in other citrate synthases are underlined. The serine residue (boldface) of *B. subtilis* ICDH corresponding to the phosphorylation site of the *E. coli* enzyme and the 26 surrounding residues identical to those of the *E. coli* enzyme are also underlined.

gene of *B. subtilis*, since *citC* mutants (such as SJB61) are defective in ICDH activity (data not shown) and since integration of plasmid pCS60 was able to transform a *citC* mutant (strain 1A32) to glutamate prototrophy.

To test whether the *citC* gene has its own promoter, plasmid pCS71, which carries an 872-bp DNA fragment containing the 3′ end of *citZ* and 5′ end of *citC* (Fig. 2), was integrated into the chromosome of *B. subtilis* JH642. The resulting strain,

TABLE 4. Sporulation frequencies of *B. subtilis* strains

Strain	Relevant genotype	No. of cells at T_{16}[a]		No. of heat-resistant cells/ total no. of viable cells	No. of heat-resistant cells/ no. of heat-resistant cells of wild type
		Total viable	Heat resistant		
JH642	Wild type	7.4×10^8	5.6×10^8	0.76 (1.00)[b]	1.00[c]
SJB33	Δ*citA*::*neo*	5.7×10^8	4.0×10^8	0.70 (0.92)	0.71
SJB66	Δ*citZ471*	1.5×10^8	3.9×10^7	0.26 (0.34)	0.07
SJB67	Δ*citA*::*neo* Δ*citZ471*	1.1×10^6	2.5×10^2	2.3×10^{-4} (3.0×10^{-4})	4.5×10^{-7}
SJB61	*citC*::*cat*	2.0×10^6	5.0×10^2	2.5×10^{-4} (3.3×10^{-4})	8.9×10^{-7}

[a] T_{16}, 16 h after end of exponential growth phase. Cells were heated for 10 min at 80°C to assay heat-resistant spores.
[b] The sporulation frequency of the wild-type strain was normalized to 1.0; the ratios of sporulation frequencies of the mutant strains to that of the wild type are shown in parentheses.
[c] The ratios of the number of heat-resistant cells of the mutant strains to that of the wild type are more likely to represent deficiencies of sporulation of the mutant strains, since the total number of viable cells of the mutant strains was underestimated because of the lysis of the cells after T_0.

SJB191, was a glutamate prototroph and sporulated well, indicating that *citC* does have its own promoter. In the accompanying paper, we show that *citC* is transcribed both from its own promoter and by read-through from the *citZ* promoter (16).

Interestingly, *B. subtilis* ICDH has a conserved Ser at the residue corresponding to the phosphorylation and substrate-binding site of *E. coli* ICDH (38) (Fig. 4). Furthermore, the 26 amino acid residues surrounding the Ser residue of *B. subtilis* ICDH are identical to those of *E. coli* ICDH (Fig. 4). *E. coli* ICDH becomes phosphorylated and inactivated when cells are grown on two-carbon compounds (such as acetate) as the sole carbon source, allowing metabolism of isocitrate through the glyoxylate shunt (38). It is possible that the catalytic activity of *B. subtilis* ICDH is inhibited by a similar covalent modification, although there is no evidence for a functional glyoxylate cycle in commonly used *B. subtilis* strains (7). A previous report described the cloning of a *B. subtilis* DNA fragment capable of complementing ICDH mutants of *B. subtilis* and *E. coli* (24). The restriction map of this cloned DNA bears no resemblance to that of the *citC* gene. We are unable to explain this discrepancy.

The putative product of the ORF downstream of the ICDH gene (whose sequence has not yet been completed) has strong similarity to malate dehydrogenases and lactate dehydrogenases. The sequence downstream of this ORF is identical to that of *phoP* (31). Strain MH5134 (from M. Hulett) has a tetracycline resistance cassette inserted at the 3′ end of the unidentified ORF; Hulett and colleagues found that this strain is partially auxotrophic for aspartate (14), a finding consistent with the notion that this ORF encodes malate dehydrogenase. Since a *citH* mutation interferes with malate dehydrogenase activity, and since the *citH* locus maps to the *phoP* region of the *B. subtilis* chromosome (13), we conclude tentatively that this ORF corresponds to *citH*.

Effect of *citA*, *citZ*, and *citC* mutations on sporulation. Mutations in *citZ* or *citC*, but not *citA*, caused a sporulation-defective phenotype on DSM plates (i.e., unpigmented, translucent colonies). As shown in Table 4, the sporulation efficiency of a strain carrying the Δ*citA*::*neo* mutation was essentially the same as that of wild-type cells, but sporulation was significantly decreased in strains carrying mutations in *citZ* or *citC*. It is noteworthy that while disruption of *citA* had little effect on sporulation, the Δ*citA*::*neo* mutation augmented substantially the sporulation defect of a *citZ* mutant strain. Since disruption of *citA* renders *citZ* mutant strains more stringently auxotrophic for glutamate and more defective for sporulation, we conclude that both *citA* and *citZ* contribute to the cell's total content of citrate synthase enzyme.

Citrate synthase, ICDH, and aconitase activities of *citA* and *citZ* mutants. Citrate synthase enzyme activity in a *citA* null mutant strain (SJB33) was within the normal range (Table 5), but this activity was significantly reduced in strains carrying mutations in *citZ* (SJB66) or in both *citA* and *citZ* (SJB67). In agreement with the notion that synthesis or activity of aconitase is dependent on citrate synthase (10), aconitase specific activity varied in parallel with citrate synthase activity. ICDH activity was slightly reduced in a nonpolar *citZ* mutant.

Under our assay conditions, strain SJB66 did not seem to have amounts of citrate synthase activity significantly above the background level despite the presence of a wild-type *citA* gene. Nonetheless, strain SJB66 was not fully auxotrophic for glutamate. It seems possible that our assay underestimates CitA enzyme activity.

In *E. coli* W620, the cloned *B. subtilis citA* gene also yielded considerably less citrate synthase enzyme activity than did the cloned *citZ* gene (Table 2). High-resolution X-ray crystallographic structures of pig heart citrate synthase and detailed analysis of other citrate synthase proteins have allowed the identification of residues important for enzyme activity (25,

TABLE 5. Enzyme activities of citrate synthase, ICDH, and aconitase of *B. subtilis* strains

Strain	Relevant genotype	Relevant phenotype	Activity[a]		
			Citrate synthase	ICDH	Aconitase
JH642	*citA*$^+$ *citZ*$^+$	Glt$^+$	1.00	1.00	1.00
SJB33	Δ*citA*::*neo*	Glt$^+$	2.10	2.00	0.93
SJB66	Δ*citZ471*	Leaky Glt$^-$	0.04	0.50	0.24
SJB67	Δ*citA*::*neo* Δ*citZ471*	Glt$^-$	0.03	0.36	0.03
SJB68	Δ*citA*::*neo* Δ*citZ471* SPβ*citZ*	Glt$^+$	1.27	1.11	1.22

[a] Enzyme specific activities of the wild-type strain were normalized to 1.00, and the ratios of enzyme activities in mutant strains to those of the wild-type strain are reported. Actual specific activities in the wild-type strain were as follows: citrate synthase, 0.3 U/mg; aconitase, 20.6 U/mg; ICDH, 82.0 U/mg. Cells were grown in DS medium to an optical density at 600 nm of approximately 1.2.

39). For example, Asp-375 is found to be conserved throughout all citrate synthase genes identified so far and to participate directly in catalysis. Mutations of Asp-375 (including a conservative substitution to Glu) dramatically reduce citrate synthase activity (17). It seemed conceivable that this is the reason why CitA (with Glu-307 instead of Asp at the corresponding catalytic site) has lower catalytic activity than does CitZ. We found that this is indeed the case. We changed Glu-307 to Asp by site-directed mutagenesis (see Materials and Methods) and found that the modified enzyme, called CitA*, is five times more active catalytically than is CitA (Table 2). CitA* and CitZ have similar enzymatic activities in *E. coli* W620 (Table 2), assuming that their rates of transcription and translation are also similar.

DISCUSSION

Our results demonstrate that *B. subtilis* has two distinct, homologous citrate synthase genes. Like citrate synthase genes from other gram-positive bacteria, *citA* and *citZ* each appear to encode a protein of about 41 kDa. This is the first direct demonstration that a single bacterial species codes for two citrate synthase enzymes. Our results indicate that the *citZ* gene is responsible for the majority of the citrate synthase activity of *B. subtilis*. There is indirect evidence that *E. coli* has two citrate synthase genes (23), and it has been suggested that *Pseudomonas aeruginosa* might have two forms of citrate synthase protein (35). However, in neither of the latter cases has the putative second citrate synthase gene been identified. It is well established that *S. cerevisiae* has two citrate synthase genes. One citrate synthase protein (Cit-II) is located in mitochondria, is involved in energy production and the utilization of nonfermentable carbon sources, and contributes 90% of total citrate synthase activity. The other citrate synthase protein (Cit-I) is located in the cytoplasm and is involved in biosynthesis of glutamate (27).

It is unclear why *B. subtilis* contains two citrate synthase genes. The two isozymes might catalyze the same condensation reaction under different metabolic conditions. It is known that total citrate synthase activity of *B. subtilis* is synergistically repressed by glucose and glutamate (8). In fact, transcription of *citZ* is strongly repressed by the combination of glucose and glutamate, but transcription of *citA*, while repressed by glucose, is stimulated by glutamate in the presence of glucose (16). Thus, *citA* might provide a basal activity when *citZ* is strongly repressed. It is also possible that the two enzymes have more specialized, nonoverlapping functions.

A temperature-sensitive mutant defective in citrate synthase activity, described by Carls and Hanson (1), was not rescued by the cloned *citA* or *citZ* gene or complemented by SPβ*citZ*, and transcriptional regulation of *citA-lacZ* and *citZ-lacZ* fusions appeared to be normal in this mutant (data not shown). In addition, by immunoblotting, the mutant was found to accumulate CitZ polypeptide in normal amounts (data not shown). Thus, the genetic and biochemical bases of this mutant remain mysterious. The mutant might be defective in citrate synthase assembly or in unknown cofactors for citrate synthase enzyme activity.

B. subtilis strains that lack citrate synthase enzyme activity have greatly reduced levels of aconitase, have slightly reduced ICDH activity, and are glutamate auxotrophs. This suggests that citrate synthase, as the first, rate-limiting enzyme of the Krebs cycle, might regulate the synthesis and function of enzymes involved in later enzymatic steps. Citrate synthase affects the function of aconitase by providing a metabolite, citrate, that controls the transcription of the aconitase gene (*citB*) (5, 10). It is unknown how citrate synthase regulates ICDH activity and whether it also regulates malate dehydrogenase activity. We are currently investigating the effects of *citA* and *citZ* mutations on the functions of *citC* and *citH* and their products.

Mutations that impair the normal function of *citA* and *citZ* result in sporulation deficiency. Our experiments in progress suggest that a *citA citZ* double mutant is blocked at an early stage but after stage 0. We are seeking to understand the basis for this block.

With the identification of the *citA*, *citZ*, *citC*, and putative *citH* genes reported here, it appears that all *B. subtilis* genes coding for Krebs cycle enzymes are known. It may now be possible to compare and contrast the mechanisms that regulate the synthesis and activity of each of these enzymes under various environmental conditions.

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