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Bacillus subtilis citB Gene Is Regulated Synergistically by Glucose and Glutamine

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The activity of aconitase in *Bacillus subtilis* is greatly reduced in cells cultured in media containing rapidly metabolized carbon sources (e.g., glucose). Thus, expression of this enzyme appears to be subject to a form of catabolite repression. Since the product of the *citB* gene of *B. subtilis* is required for aconitase activity, we cloned the wild-type allele of this gene and used this DNA as a probe for transcription of *citB* in cells grown in various media. The steady-state level of RNA that hybridized to this probe was about 10-fold higher in *B. subtilis* cells grown in citrate-glutamine medium than in cells grown in glucose-glutamine medium. This result correlates well with the steady-state levels of aconitase activity. Two transcripts were shown to initiate within the cloned DNA; the steady-state level of one of these transcripts varied in the same way as did aconitase activity when cells were grown in media containing different carbon sources. This is the first demonstration of regulation by the carbon source of the level of a vegetative-cell transcript in *B. subtilis*.

In *Bacillus subtilis*, the first three enzymes of the citric acid cycle, i.e., citrate synthase, aconitase, and isocitrate dehydrogenase, are required both for biosynthesis of glutamate (through 2-ketoglutarate) and for utilization of nonfermentable energy sources (e.g., citrate, lactate, and various amino acids). The levels of these citric acid cycle enzymes in *B. subtilis* are reduced about twofold in the presence of a rapidly metabolized carbon source such as glucose (27, 28, 43). The residual level of activity apparently suffices for biosynthesis of amino acids and other compounds. The levels of citrate synthase (18, 28, 29), aconitase (27, 28, 51), and, in some strains, isocitrate dehydrogenase (19, 29, 43) are further reduced when a rapidly metabolized carbon source and a source of glutamate are both supplied. Glutamate alone or in combination with a poor carbon source (e.g., citrate) does not lead to reduction in the levels of these enzymes (28).

Unlike the situation with *Escherichia coli*, regulation of carbon source utilization in *Bacillus* spp. does not appear to involve cyclic AMP (2, 6, 30, 49) or the phosphoenolpyruvate phosphotransferase sugar transport system (33). Attempts to isolate pleiotropic mutants that would have phenotypes equivalent to those of mutants of catabolite gene activator protein in *E. coli* (9) have not yet been successful (7, 13–15). Moreover, regulation by the carbon source of the levels of various vegetative enzymes in *B. subtilis* may not be governed by a single mechanism. For example, accumulation of only the initial phosphorylated intermediates of various readily utilized sugars is sufficient for reduction of acetoin dehydrogenase levels (33) but not for regulation of the levels of inositol dehydrogenase (22) or D-gluconate transport enzymes (10).

To explore the mechanism of synergistic regulation of aconitase levels by glucose and glutamate, we have isolated a fragment of *B. subtilis* DNA which transforms an aconitase-deficient strain, 1A120 (*citB75*), to wild type. Using a 1.1-kilobase-pair (kb) subclone which retains transforming activity as a hybridization probe, we have demonstrated that the steady-state level of a transcript initiating in the *citB* region is regulated similarly to aconitase specific activity. This transcript appears to be necessary for aconitase activity. Regulation by the carbon source of the level of any transcript in *B. subtilis* has not been demonstrated previously.

MATERIALS AND METHODS

Bacterial strains and cloning vectors. *E. coli* MM294 (*endA hsdR thi pro*; supplied by R. Losick) was used as cloning host. Strain DP50 *supF hsdS dapD8 lacY* Δ(*gal-uvrB*) Δ*thyA gyrA supE44 supF48*; obtained from J. Hoch) was used to propagate recombinant lambda phages containing *B. subtilis* DNA. All recombinant plasmids were derived from pBR325 (4, 46), which was supplied by K. Dharmalingham. Competent *E. coli* cells were prepared by the method of Davis et al. (8) and were frozen, thawed, and transformed as described by Morrison (40).

B. subtilis SMY (wild type) was obtained from R. Losick, and aconitase-deficient strains 1A120 (*citB75 trpC2*) and 1A331 (*citB1 trpC2*) were obtained from the Bacillus Genetic Stock Center. *B. subtilis* strains were grown to competence and transformed with DNA as described by Dubnau and Davidoff-Abelson (11).

Propagation of a *B. subtilis* gene bank and isolation of phage DNA. A *B. subtilis* gene bank consisting of *Eco*RI fragments cloned in λgtWES.λB was obtained from J. Hoch. Recombinant lambda phages were propagated as described by Fisher et al. (16). Large-scale plasmid preparations were made by the method of Birnboim and Doly (3). Plasmid DNA was purified by equilibrium centrifugation in CsCl gradients containing 400 μg of ethidium bromide and 1 g of oven-dried CsCl per ml. Restriction enzymes and T4 DNA ligase were obtained from New England BioLabs, Inc., or Bethesda Research Laboratories, Inc., and reactions were carried out in the buffers described by Maniatis et al. (36). For cloning experiments, DNA fragments to be inserted were supplied at a threefold molar excess relative to the vector, and the total DNA concentration was 50 to 150 μg/ml.

Enzyme assays. Extracts were prepared by the procedure

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TABLE 1. Enzyme activities in a *citB* mutant

Strain	Aconitase sp act[a]	Isocitrate dehydrogenase sp act[b]
1A120 (*citB75*)	6.1	307
LDD1 (*cit*$^+$)[c]	40.3	215
1A120A (*cit*$^+$)[d]	32.7	
BR151 (*cit* $^+$)	146.2	
JAD7 (citB84)[e]	9.5	

[a] Aconitase specific activity (nanomoles of *cis*-aconitate produced per minute per milligram of protein at 22°C) was determined (see Materials and Methods) in extracts of cells grown in DSM medium (17) and harvested 1.5 h after the end of growth.

[b] Isocitrate dehydrogenase specific activity (micromoles of NADPH produced per minute per milligram of protein at 22°C) was determined (see Materials and Methods) in extracts of cells grown in TSS minimal medium containing glutamine and limiting (0.07%) glucose and harvested 0.5 h after the end of growth.

[c] Strain LDD1 was derived from strain 1A120 by transformation to glutamate prototrophy with chromosomal DNA of strain SMY.

[d] Strain 1A120A was derived from strain 1A120 by transformation to glutamate prototrophy with plasmid pMR41.

[e] Strain JAD7 carries a *citB* mutation generated by in vitro mutagenesis of cloned *citB* DNA (see Materials and Methods).

of Fortnagel and Freese (20). Aconitase and isocitrate dehydrogenase activities were determined as described by Hanson and Cox (28). Units of aconitase activity (nanomoles of *cis*-aconitate produced per minute from isocitrate at 22°C) were based on a molar extinction coefficient for *cis*-aconitate at 240 nm of 3.3×10^3 cm^{-1} M^{-1}. Units of isocitrate dehydrogenase activity (nanomoles of NADPH produced per minute from NADP at 22°C) were based on a molar extinction coefficient for NADPH at 340 nm of 6.2×10^3 cm^{-1} M^{-1}. Protein concentration was determined by the Bio-Rad assay, with bovine serum albumin (fraction V; Miles Laboratories, Inc.) as the standard.

Growth of *B. subtilis* for physiological studies. Dilutions of wild-type strain SMY were grown overnight in TSS medium (50 mM Tris hydrochloride [pH 7.5], 40 μg of $FeCl_3$-sodium citrate per ml, 2.5 mM K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.5% glucose or 0.5% sodium citrate or both, and 0.2% NH_4Cl or 0.2% glutamine which was freshly dissolved in 100 mM Tris hydrochloride [pH 7.0] and filter sterilized). Overnight cultures still in log phase were pelleted, washed with 50 mM Tris hydrochloride (pH 7.5), and suspended in fresh medium to give a turbidity reading (Klett-Summerson colorimeter; green filter) of about 15 for glucose cultures (doubling time was approximately 50 min) or about 40 for citrate cultures (doubling time was 90 to 150 min). Cells were harvested at mid-log phase (100 to 150 Klett units), and cell pellets were rapidly frozen in dry ice and stored at −70°C for subsequent isolation of RNA and determination of aconitase specific activity.

Isolation of RNA from *B. subtilis*. RNA was isolated from frozen cell pellets and purified by the method of Ollington et al. (44). Cells were lysed with lysozyme and then treated with sodium dodecyl sulfate and diethyl pyrocarbonate. Separation of mRNA and rRNA from tRNA and DNA was achieved by elution with ethanol-water solutions from a cellulose column (21, 44). Alternatively, RNA was isolated by the method of Zuber and Losick (56). Cells were opened in the presence of guanidinium isothiocyanate in a French pressure cell, and RNA was precipitated selectively in the presence of 2 M lithium chloride. RNA was stored at −20°C in 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA containing 67% ethanol and was mixed well immediately before removal of samples.

Dot blot hybridizations. Dot blots were performed by the method of Thomas (52). DNA was labeled with a ^{32}P-nucleotide to a specific activity of approximately 10^7 cpm/μg by the nick translation activity of *E. coli* DNA polymerase I (36).

End labeling of DNA. Restricted DNA was treated with calf intestinal alkaline phosphatase and T4 polynucleotide kinase (DNA containing 3′ overhanging or blunt ends was denatured before each reaction) as described by Maxam and Gilbert (38), except that DNA was microdialyzed before kinase treatment against 10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA on Millipore VMWP filters (37) to remove any traces of ammonium sulfate. For some S1 mapping experiments, end-labeled DNA was restricted with a second enzyme, and a single end-labeled DNA fragment was isolated from a polyacrylamide gel (38). To label the 5′ ends of *Pst*I sites, 3′ overhanging DNA ends were first converted to blunt ends by treatment with T4 DNA polymerase (36).

Localization of transcriptional initiation sites. S1 nuclease mapping was performed by the method of Berk and Sharp (1), except that RNA mixed with ^{32}P-labeled 5′-end-labeled DNA was frozen in crushed dry ice and lyophilized to dryness (rather than being coprecipitated with ethanol) before suspension in 10 μl of hybridization buffer. Approximately 10,000 cpm of DNA which had been labeled with ^{32}P at one or both ends was hybridized with 0.1 to 200 μg of *B. subtilis* RNA. Quantitative S1 mapping was performed by varying the amount of *B. subtilis* RNA added to an excess of DNA. All samples were brought to the same final RNA concentration by the addition of *Saccharomyces cerevisiae* RNA before incubation for hybridization. S1-protected fragments were electrophoresed as single-stranded fragments in 5% polyacrylamide–8 M urea gels (36).

Quantitation of in vivo transcripts. S1-protected fragments were located by autoradiography (as in Fig. 6 and 7), and these bands were excised. Gel slices were immersed in toluene-Omnifluor, and radioactivity was determined by averaging several 10-min periods of scintillation counting. The values obtained were corrected for background (about 20 cpm). Background measured in gel slices from just above or just below the bands varied with the intensity of the band (20 to 50 cpm), and correction of the values by subtraction of these backgrounds did not significantly alter the results. Threefold increases in the amount of RNA used gave threefold increases in measured radioactivity in the protected band, demonstrating that the hybridization probe was in excess.

In vitro transcription. Run-off transcripts were detected by the incubation of restricted plasmid DNAs with RNA polymerase in a standard in vitro transcription reaction mix (16). The $E\sigma^{55}$ form of *B. subtilis* RNA polymerase was prepared by our published method (32). the $E\sigma^{32}$ and $E\sigma^{37}$ forms of RNA polymerase were gifts of C. Binnie, and the $E\sigma^{29}$ form was provided by W. Haldenwang. Transcription products were analyzed by electrophoresis in 6% gels of polyacrylamide containing 8 M urea, followed by autoradiography.

In vitro mutagenesis. Plasmid pMR41 was digested with *Hin*dIII,and unpaired 5′ extensions were removed by treatment with nuclease S1. After incubation with T4 DNA ligase, the DNA was exposed to *Hin*dIII again to linearize any molecules that still retained an intact *Hin*dIII site. This mixture was used to transform *E. coli* MM294, selecting for chloramphenicol resistance. Transformants were screened for plasmids lacking a *Hin*dIII site. One such plasmid was named pDWD2 and was shown by restriction mapping to be indistinguishable from pMR41 except for the lack of a *Hin*dIII site. To introduce the mutated version of the *citB*

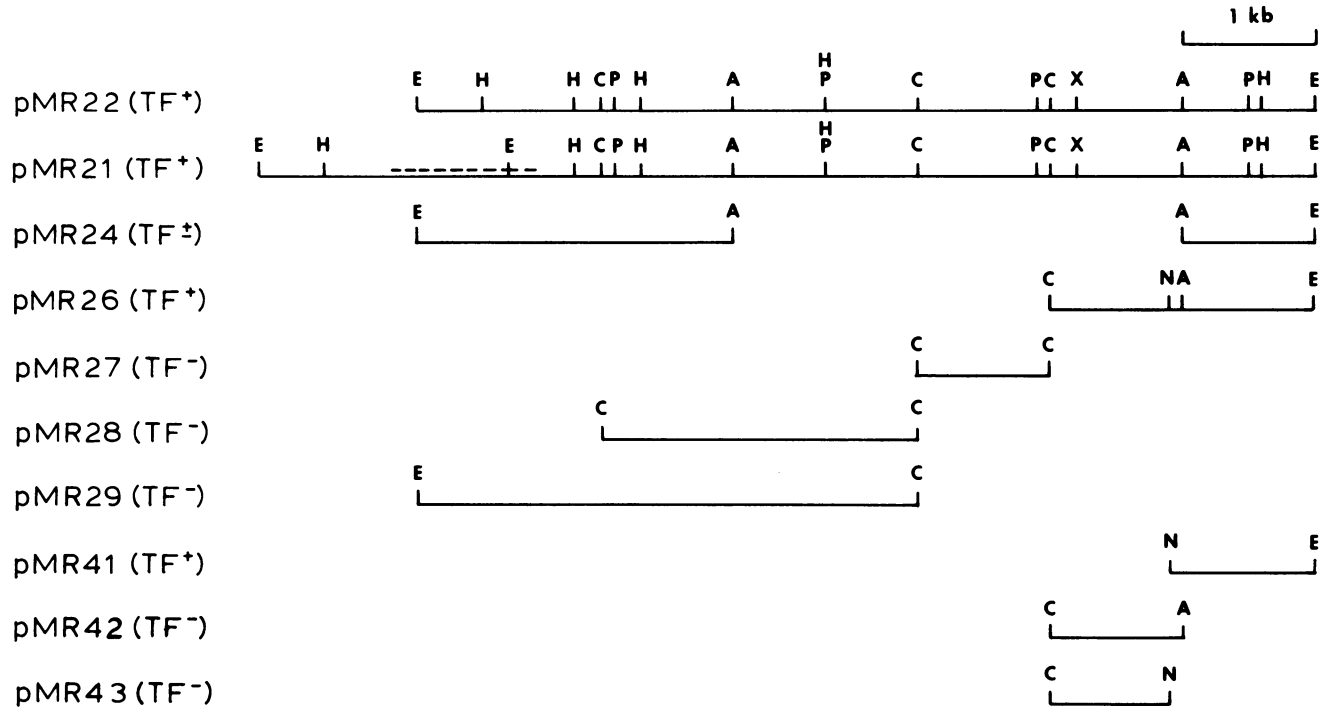

FIG. 1. Cloned *B. subtilis* DNA from the region of *citB*. Various fragments from the region of the *citB* locus were subcloned in successive steps from a λgt WES.λB phage that had CitB$^+$-transforming activity. Plasmids able to transform *citB75* to *citB*$^+$ are designated TF$^+$. Vector DNA (pBR325) is not shown. Restriction maps are drawn to the scale indicated. Restriction site abbreviations are as follows: A, *Aval*; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nde*I; P, *Pst*I; X, *Xba*I. The dotted horizontal line indicates the approximate location of a 1.1-kb spontaneous insertion of DNA present in pMR21.

region into the *B. subtilis* chromosome, pDWD2 DNA was linearized with *Pvu*II (the single *Pvu*II site of pDWD2 is within vector DNA) and mixed with chromosomal DNA of strain SMY, and the mixture was used to transform strain BR151 (*trpC2 metB10 lys-3*) to Trp$^+$. Among 180 Trp$^+$ transformants, 2 required a source of glutamate for growth. One such strain was designated JAD7 and was shown to be lacking in aconitase activity (Table 1).

RESULTS

Isolation of recombinant λ phages containing the *citB* locus of *B. subtilis*. The λ Charon 4A and λgtWes.λB *B. subtilis* chromosome banks of Ferrari et al. (12) were assayed for ability to transform aconitase-deficient mutants 1A120 (*citB75*) and 1A331 (*citB1*) to wild type (glutamate prototrophy was selected). While other mutations causing aconitase deficiency exist (5, 20, 47), only these two mutations have been mapped (55). Both of these *citB* mutations map near the terminus of DNA replication, between *gltA* and *glnA*.

Although both strains became competent for transformation, CitB$^+$-transforming activity by the λ banks was detected only for strain 1A120 and only with the λgtWES.λB bank. Subpools of this bank were tested, and two *citB*-transforming phages were isolated. Both phages (and various plasmid subclones; see below) transformed *citB75*, but not *citB1*, to wild type, and both phages contained a single *Eco*RI fragment of about 7 kb of *B. subtilis* DNA.

Subcloning *citB* in plasmid pBR325. The 7-kb *Eco*RI fragment of one of the λ phages was cloned into the *Eco*RI site of the *E. coli* vector pBR325, eliminating chloramphenicol resistance and creating pMR22. A restriction map of pMR22 is presented in Fig. 1. Of various subclones constructed from pMR22, only those plasmids which retained the right-hand end of the 7-kb *Eco*RI fragment retained CitB$^+$-transforming activity (Fig. 1). In these experiments, only recombination was assayed. We have not determined whether the 7-kb *Eco*RI fragment complements *citB75* in *trans*.

The mutation *citB75* affects aconitase activity. Since the *citB* locus was cloned solely on the basis of restoration of glutamate prototrophy in strain 1A120 (*citB75*), it was important to confirm that this strain was specifically deficient in aconitase activity rather than in glutamate synthase, citrate synthase, or isocitrate dehydrogenase activity. We first showed that strain 1A120 in glutamine-containing medium, unlike a CitB$^+$ strain, is unable to utilize lactate or citrate as sole carbon source (data not shown), eliminating the possibility of a defect solely in glutamate synthase or citrate synthase activity. Therefore, aconitase and isocitrate dehydrogenase activities in strain 1A120 were measured (Table 1). Both strain 1A120 (*citB75*) and a transformant to glutamate prototrophy, strain LDD1, had high levels of isocitrate dehydrogenase activity, but only LDD1 had high levels of aconitase activity. Therefore strain 1A120 (*citB75*) is specifically defective in aconitase activity. Transformation of strain 1A120 to glutamate prototrophy by plasmid pMR41 also led to restoration of aconitase activity (Table 1).

Level of RNA hybridizing to cloned *citB* region DNA is regulated by the carbon source. To determine whether the level of *citB* region RNA is regulated by the carbon source in a manner similar to that seen for aconitase activity, dot blot hybridizations were performed. RNA was isolated from strain SMY grown in citrate-glutamine (high aconitase levels) or glucose-glutamine (low aconitase levels) medium and

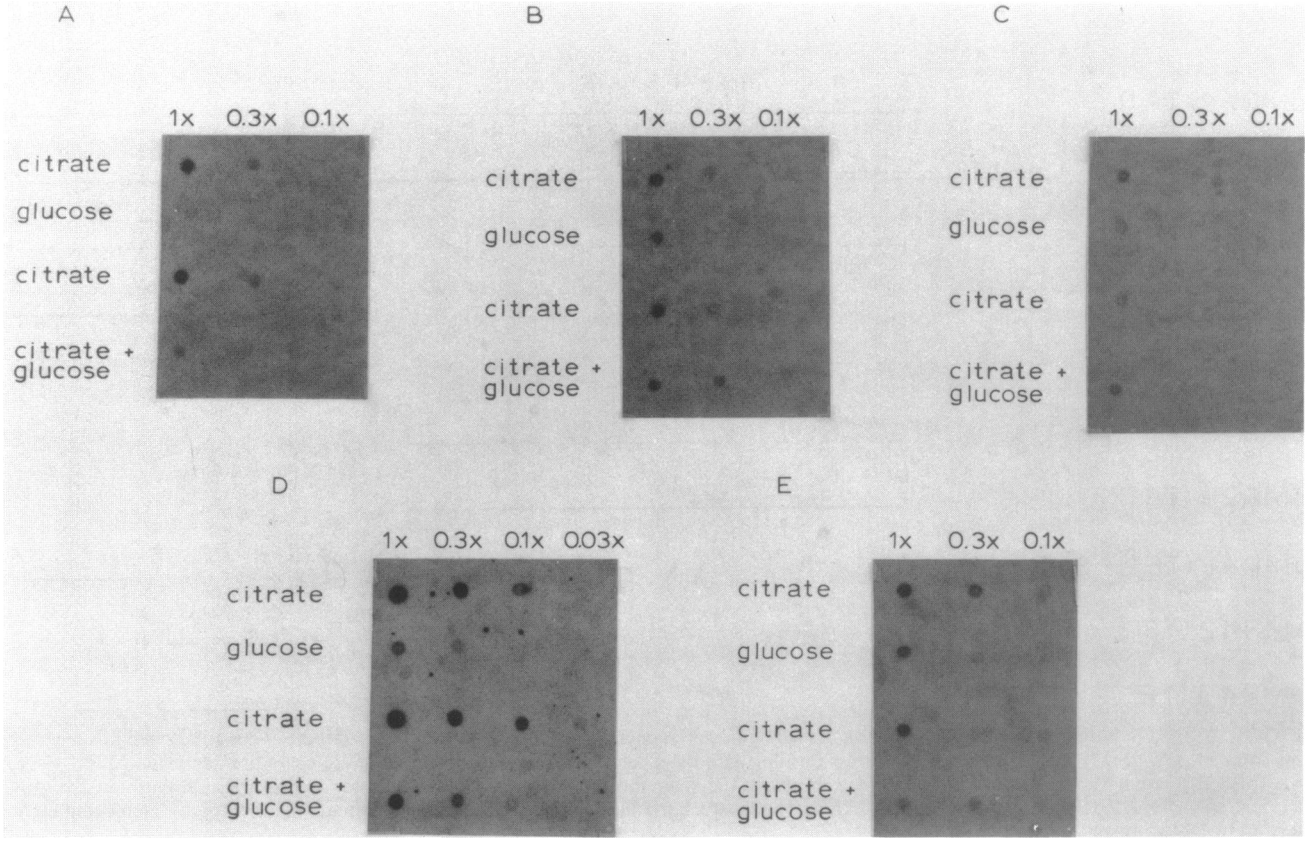

FIG. 2. Dot blot hybridizations of RNA to ^{32}P-labeled *citB* region DNA. RNA was isolated from *B. subtilis* SMY grown in TSS medium containing glutamine plus citrate or glucose or both as the carbon source. Samples (5 μl) of threefold dilutions of RNAs were applied to sheets of nitrocellulose. The maximum amount of RNA spotted was in each case 2.5 μg. After being baked at 80°C for 2 h, the sheets were incubated with denatured plasmid DNA that had been labeled with ^{32}P by nick translation. After hybridization, the blots were washed and subjected to autoradiography. The labeled DNA probes were pMR26 (A), pMR28 (B), pMR27 (C), pMR41 (D), and pMR43 (E).

hybridized to ^{32}P-labeled plasmid subclones of *citB* region DNA (Fig. 2).

RNA hybridizing to pMR27 and pMR28, which contain DNA to the left of the *citB* region (Fig. 1), was present at a nearly constant level in cells grown in various media. Therefore, either the transcripts from these regions are not regulated by the carbon source, or various transcripts are regulated in opposing fashion, yielding no net appearance of regulation.

TABLE 2. Aconitase activity in cells grown in different media

Medium[a]	Aconitase sp act[b]	
	Expt 1	Expt 2[c]
Citrate-ammonia	ND	422
Citrate-glutamine	400	328
Glucose-ammonia	326	339
Glucose-glutamine	13	40
Citrate-glucose-ammonia	171	176
Citrate-glucose-glutamine	19	37

[a] *B. subtilis* SMY was grown to mid-log phase in TSS medium modified to contain only the carbon and nitrogen sources listed.

[b] Aconitase specific activity was determined as described in Materials and Methods and is expressed as nanomoles of *cis*-aconitate produced per minute per milligram of protein at 22°C. ND, Not determined.

[c] The cultures used for enzyme assays in this experiment were the sources of the RNA used in the S1 mapping experiments shown in Fig. 6 and 7.

In contrast, the steady-state level of RNA which hybridized to plasmid pMR26 varied with the carbon source. We estimate from these dot hybridization experiments that the relative level of RNA hybridizing to pMR26 is 10-fold higher in strain SMY in citrate-glutamine medium than in glucose-glutamine medium. Aconitase specific activity in the same strain in these media varies by 10 to 20-fold (Table 2). Plasmid pMR41, a subclone of pMR26 (Fig. 1), retains 1.1 kb of *B. subtilis* DNA and CitB$^+$-transforming activity. The steady-state level of RNA hybridizing to pMR41 was also about 10-fold higher in cells grown in citrate-glutamine medium than in cells grown in glucose-glutamine medium. Unlike the case for RNA hybridizing to pMR26, RNA hybridizing to pMR41 seemed to be about threefold more abundant when citrate was added to glucose-glutamine medium. Ohne (42) has suggested that citrate acts as an inducer of aconitase.

Plasmid pMR43 contains the remaining 1 kb of *B. subtilis* DNA cloned in pMR26 (Fig. 1) and does not retain CitB$^+$-transforming activity. RNA hybridizing to pMR43 was present at a constant level in strain SMY grown in various media.

In summary, the steady-state levels of one or more transcripts coded for by the DNA region having CitB$^+$-

transforming activity are regulated by the carbon source in a manner similar to that for aconitase specific activity.

Localization of transcription initiation sites within the *citB* region. Transcription initiation sites within the *citB* region were identified by S1 nuclease mapping experiments (1). *B. subtilis* RNA was isolated from strain SMY grown in citrate-glutamine or glucose-glutamine medium and hybridized to pMR26 or pMR41, which had been restricted at one of several sites and labeled with [^{32}P]phosphate at its 5′ ends.

Two transcription initiation sites were located within the 1.1 kb of *B. subtilis* DNA cloned in pMR41. When either pMR26 or pMR41 was restricted with *Ava*I and labeled at the 5′ ends, a protected fragment of about 270 bases (Fig. 3) was detected. This protected fragment was obtained only with RNA isolated from cells grown in citrate-glutamine medium. Since this protected fragment was also produced with a purified *Ava*I-*Pst*I fragment labeled only at the *Ava*I site (Fig. 3B), transcript I must be synthesized in the right-to-left direction (Fig. 4), starting about 270 base pairs (bp) to the right of the *Ava*I site located in the region of CitB$^+$-transforming activity.

A second transcription initiation site was located 200 bp from a *Hin*dIII site and 90 bp from a *Pst*I site (Fig. 5), again only with RNA from cells grown in citrate-glutamine medium. When DNA labeled at the 5′ ends at the *Pst*I sites was restricted with *Hin*dIII, the 90-base protected fragment still appeared after hybridization and S1 treatment. Since the distance between these sites is about 110 bp (Fig. 5B, and data not shown) it was likely and is proved below that these various protected fragments were derived from one transcript starting at 90 bp to the left of the *Pst*I site and 200 bp to the left of the nearest *Hin*dIII site (transcript II, Fig. 4). To determine unambiguously the location and direction of this transcript, pMR26 DNA was labeled at the 5′ ends of the *Bgl*II site (located about 180 bp downstream of the nearest *Hin*dIII site) and subsequently restricted with *Rsa*I. A 450-bp probe (Fig. 5C) was purified from an acrylamide gel. With this probe, a 380-base protected fragment was observed (Fig. 5C), showing that transcript II is oriented as shown in Fig. 4. The *Eco*RI end of the cloned *citB* DNA lies only about 180 bp downstream of this *Bgl*II site; we have not determined whether transcript II continues up to or beyond the end of the cloned *citB* DNA.

In summary, at least two transcripts are initiated within the 1.1-kb region which transforms *citB75* to wild type. The levels of both transcripts, like the levels of aconitase and the level of RNA hybridizing to pMR26 and pMR41 (Fig. 2), are higher in cells grown in citrate-glutamine medium than in cells grown in glucose-glutamine medium.

Synergistic regulation of transcript II. Proof that the level of transcript I or II is regulated in a manner similar to the regulation of the level of aconitase specific activity requires a demonstration of synergistic regulation of these transcripts by the combination of glucose and a source of glutamate (e.g., glutamine). Therefore, S1 mapping was performed with RNA that had been isolated from strain SMY grown in six different minimal media (citrate-ammonia, citrate-glutamine, glucose-ammonia, glucose-glutamine, citrate-glucose-ammonia, and citrate-glucose-glutamine). The level of transcript II (detected as a 200-base protected fragment of pMR41 labeled at the 5′ ends of the *Hin*dIII site; Fig. 6) was similar in cells grown in all media tested except for those containing both glucose and glutamine; in the latter case, the level of transcript II was reduced at least 9-fold (aconitase was reduced 10- to 20-fold; Table 2).

The relative levels of transcript II were quantitated by excising the S1-protected fragments from gels and determining radioactivity by liquid scintillation counting. In one such experiment (Fig. 7), the steady-state level of transcript II (relative to total RNA) was 14-fold higher in cells grown in citrate-glutamine medium than in cells grown in glucose-glutamine medium. In a duplicate experiment, the difference was 20-fold.

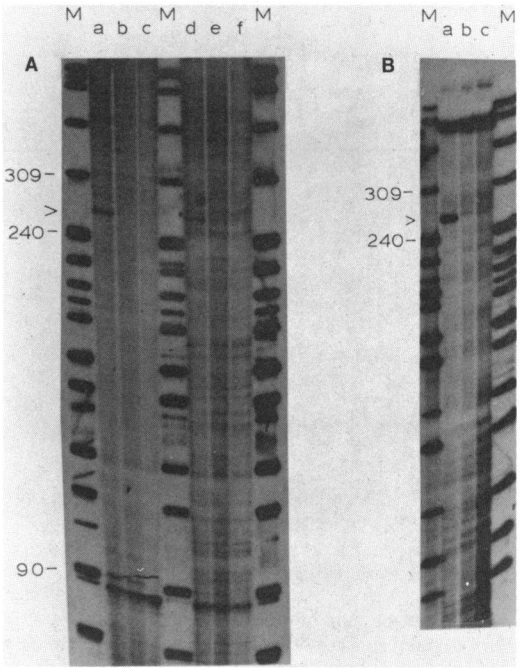

FIG. 3. Identification of *citB* region transcipt I by S1 nuclease mapping. RNA was isolated from *B. subtilis* SMY grown in citrate-glutamine or glucose-glutamine minimal medium, and equal amounts of RNA were hybridized with ^{32}P-labeled 5′-end-labeled *Ava*I fragments of various plasmids carrying DNA from the *citB* region. Hybridized samples were treated with S1 nuclease, denatured, and electrophoresed on urea-polyacrylamide gels with pBR322-*Hpa*II size markers (M). Sizes of fragments are indicated in bases. (A) In lanes a through c, the probe was pMR26 labeled at *Ava*I ends. RNA sources were citrate culture (a), glucose culture (b), and *S. cerevisiae* (c). In lanes d through f, the probe was pMR41 labeled at *Ava*I ends. RNA sources were citrate culture (d), glucose culture (e) and *S. cerevisiae* (f). (B) The probe was a purified 500-bp *Ava*I-*Pst*I fragment (labeled only at the *Ava*I site). RNA sources were citrate culture (a), glucose culture (b), and *S. cerevisiae* (c).

As a control for the intactness and hybridizability of the various RNA samples, these samples were simultaneously probed for the *veg* gene transcript (45). Little is known about expression of the *veg* gene other than that it is expressed only during growth and early in sporulation (23, 45). While expression of the *veg* gene in various media has not been extensively characterized, there was no reason to suspect that this gene would be subject to regulation under the growth conditions tested, and no more suitable control was available. For S1 mapping, plasmid pPH9, a pBR322 derivative that contains the *veg* promoter region (P. Hopper, S. F. J. LeGrice, and A. L. Sonenshein, unpublished data) was restricted and 5′ end labeled at its *Bam*HI site. In contrast to the strong regulation in the level of transcript II, the amount of the 100-base protected fragment produced by the *veg* transcript varied in cells grown in different media by no more than twofold (Fig. 6).

Regulation of transcript I. Transcript I was detected at

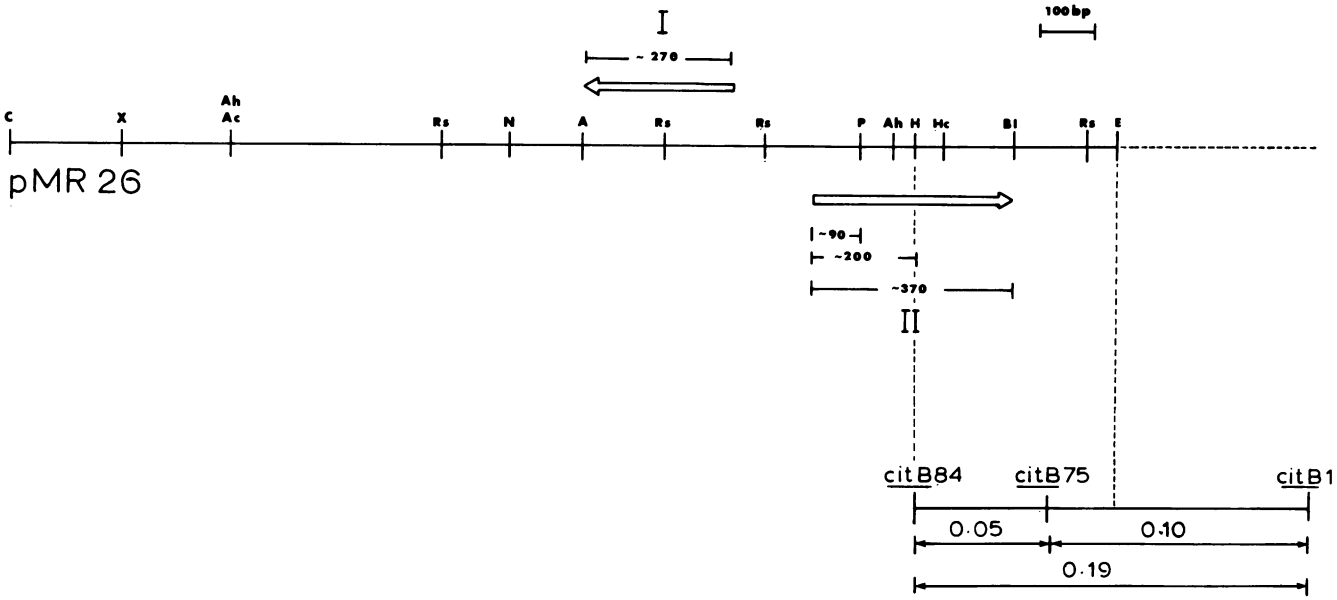

FIG. 4. Restriction map of *citB* region with locations of in vivo transcription initiation sites. Distances between restriction sites within the insert DNA of pMR41 were determined by polyacrylamide gel electrophoresis of restriction fragments and are drawn to the scale indicated. Restriction sites are abbreviated as in Fig.1. Additional abbreviations: Ac, *Acc*I; Ah, *Aha*III; Rs, *Rsa*I; Hc, *Hin*cII; and Bl, *Bgl*II. The arrows indicate the initiation sites, directions of synthesis, and minimum lengths of *citB* region transcripts (as determined by S1 nuclease mapping). Locations of various *citB* mutations are indicated in the lower part of the figure. Distances shown between mutations are based on measurements of recombination index, with the mutation *trpC2* as a reference marker.

higher levels in cells grown in citrate-glutamine medium than in cells grown in glucose-glutamine medium (Fig. 3). However, the level of this transcript varied in different batches of RNA. In related work, we have shown that the level of transcript I (unlike that of transcript II) increases dramatically at an intermediate time during sporulation in a complex medium (Dingman, Rosenkrantz, and Sonenshein, manuscript in preparation). It is possible that synthesis of transcript I is a sporulation-dependent function that is turned on at low levels or in a fraction of the population growing slowly on a poor carbon source. A typical culture growing in citrate-glutamine medium contained 0.1% of the population as heat-resistant spores.

In vitro synthesis of *citB* region transcripts. Growing and sporulating cells of *B. subtilis* are known to contain several forms of RNA polymerase (23, 31, 34, 54). These RNA polymerases appear to differ only in the sigma factor associated with a common core unit. Each sigma factor directs recognition of a class of promoters, which appear to contain sequences in common centered about 10 and 35 bp upstream from the RNA start point (23, 34, 39).

The major vegetative ($E\sigma^{55}$) form of *B. subtilis* RNA polymerase, containing a sigma factor with an apparent molecular weight of 55,000 (actual M_w, 43,000 [24]), generated a 200-base run-off transcript with *Hin*dIII-cut pMR41 and a 105-base naturally terminated transcript from vector pBR325 (Fig. 8, lanes a and d). (This latter transcript is from the origin of plasmid replication; S. F. J. LeGrice, personal communication.) The 200-base run-off transcript was indistinguishable in size from the S1-protected fragment generated with in vivo RNA and *Hin*dIII-cut pMR41 (lane b). This run-off transcript also appears to be synthesized in the same

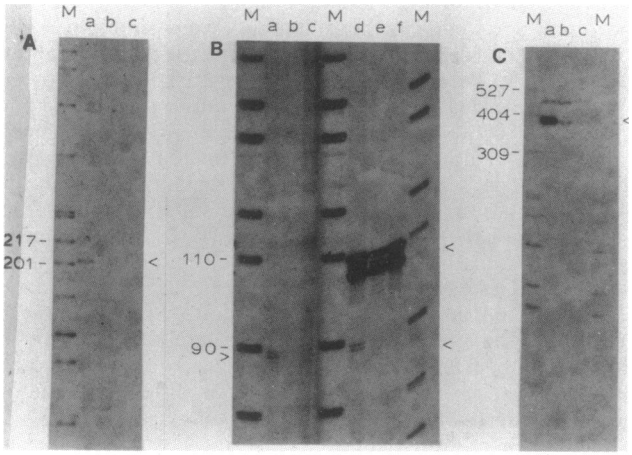

FIG. 5. Identification of *citB* region transcript II by S1 nuclease mapping. RNA was isolated from *B. subtilis* SMY grown in citrate-glutamine or glucose-glutamine minimal medium, and equal amounts of RNA were hybridized with ^{32}P-labeled 5′-end-labeled restriction frgments of various plasmids carrying *citB* region DNA. Hybridized samples were treated with S1 nuclease, denatured, and electrophoresed on urea-polyacrylamide gels with pBR322-*Hpa*II size markers (M). Sizes of fragments are indicated in bases. (A) The probe was pMR26 labeled at *Hin*dIII ends. RNA sources were citrate culture (a), glucose culture (b), and *S. cerevisiae* (c). (B) The probe was pMR26 labeled at *Pst*I ends. In lanes d through f, the DNA was subsequently digested with *Hin*dIII. RNA sources were citrate culture (a and d), glucose culture (b and e), and *S. cerevisiae* (c and f). The family of bands migrating at 105 to 110 bases in lanes d through f is undigested probe DNA. (C) The probe was a 450-bp *Bgl*II-*Rsa*I fragment labeled at the *Bgl*II end. RNA sources were citrate culture (a), glucose culture (b), and *S. cerevisiae* (c).

direction across the *Hin*dIII site as in vivo transcript II, since restriction of pMR41 with *Pst*I (lane c) produced a template which gave the expected run-off transcript of about 90 bases. Furthermore, restriction of pMR41 with *Bgl*II and *Rsa*I gave templates for transcripts of 380 and 500 bases, respectively (data not shown).

Synthesis of this 200-base run-off to the *Hin*dIII site of pMR41 was not detected with either a sporulation-specific form of RNA polymerase ($E\sigma^{29}$; 25) containing a sigma factor of 29,000 daltons (lane e) or with the minor vegetative forms of RNA polymerase containing sigma factors of 37,000 ($E\sigma^{37}$; lane h) or 32,000 ($E\sigma^{32}$; data not shown) daltons which were active with the promoters for the *spoVG* gene (lanes f and g; 26, 31).

These data suggest that the major vegetative ($E\sigma^{55}$) form of RNA polymerase is responsible for synthesis of transcript II in vivo. The decrease in this form of RNA polymerase early during sporulation (35, 53) correlates with the disappearance of transcript II (Dingman et al., in preparation). None of the forms of RNA polymerase tested ($E\sigma^{55}$, $E\sigma^{37}$, $E\sigma^{32}$, and $E\sigma^{29}$) was able to direct synthesis of an in vitro transcript corresponding to the in vivo transcript I (data not shown).

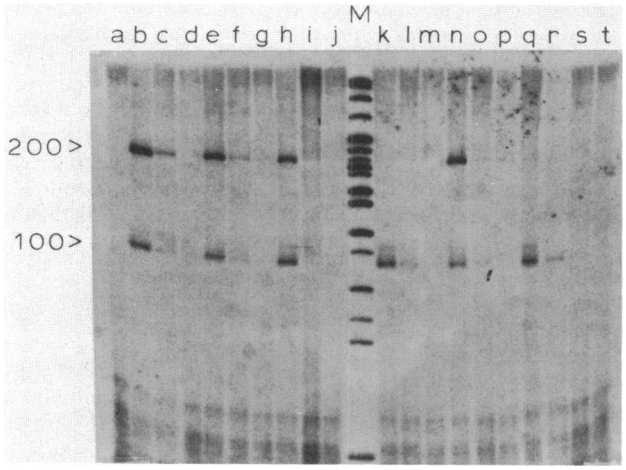

FIG. 6. Levels of *citB* region transcript II under six growth conditions. RNA was isolated from *B. subtilis* SMY grown in media containing citrate or glucose or both (as the carbon source) and ammonia or glutamine (as the nitrogen source). Threefold dilutions of RNA (all brought to a total of 9 μg by supplementation with *S. cerevisiae* RNA) were hybridized with pMR41 that had been 5′ end labeled with [^{32}P]phosphate at the *Hin*dIII site and with pPH9 that had been 5′ end labeled at the *Bam*HI site. Plasmid pPH9 (P. Hopper, personal communication) is a derivative of pMS480 (M. Stephens and R. Losick, personal communication) and contains a *Bam*HI linker at the *Hae*III site located approximately 100 bp downstream of the transcription initiation site of the *veg* gene (45). Despite the potential lack of homology of this linker with the native sequence, *veg* RNA protected label at the *Bam*HI site (although the hybridization temperature had to be reduced from the standard 50 to 45°C for efficient hybridization in 80% formamide). Hybridized samples were treated with S1 nuclease, denatured, and electrophoresed on urea-polyacrylamide gels with pBR322-*Hpa*II size markers (M). Sizes of fragments are indicated in bases. Sources of RNA and micrograms used were as follows: *S. cerevisiae*, 9 (a); citrate-ammonia culture, 9 (b); 3 (c), and 1 (d); citrate-glutamine culture, 9 (e), 3 (f), and 1 (g); glucose-ammonia culture, 9 (h), 3 (i), and 1 (j); glucose-glutamine culture, 9 (k), 3 (l), and 1 (m); citrate-glucose-ammonia culture, 9 (n), 3 (o), and 1 (p); citrate-glucose-glutamine culture, 9 (q), 3 (r), and 1 (s); *S. cerevisiae*, 9 (t).

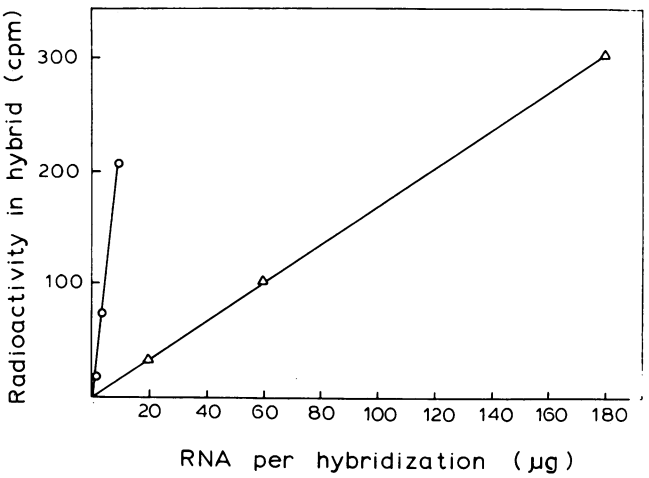

FIG. 7. Quantitation of levels of *citB* region transcript II. Bands corresponding to the 200-base protected fragment seen in Fig. 6 were excised from a similar gel and suspended in scintillation fluid. Radioactivity determined for each sample was the average of several 10-min counting periods. Samples were of RNA from a citrate-glutamine culture (○) and a glucose-glutamine culture (△).

In vitro generation of a mutation in transcript II. The experiments described above demonstrate that the level of transcript II is regulated similarly to the level of aconitase activity. However, it was possible to test directly whether transcript II is necessary for aconitase activity and whether the *citB75* mutation lies within the region coding for transcript II. A mutation was generated in vitro in the region of pMR41 encoding transcript II by restricting with *Hin*dIII and treating with nuclease S1 to remove unpaired bases (see Materials and Methods). If the *Hin*dIII site lies within a protein-coding region of transcript II, a frameshift mutation should have occurred. The altered region encoding transcript II was then substituted for the chromosomal copy by transformation of strain BR151 by the mutated pMR41 mixed with chromosomal DNA of strain SMY. Trp$^+$ transformants were selected to enrich for competent cells in the population and were screened for the CitB$^-$ phenotypes (glutamate or glutamine auxotrophy, inability to utilize citrate for energy, lack of aconitase activity [Table 1], and inability to sporulate). Such mutants were isolated, suggesting that replacement of the wild-type region encoding transcript II led to introduction of a *citB* mutation (*citB84* in Fig. 4). Chromosomal DNA of isogenic Cit$^+$ and *citB84* mutant strains was digested with *Hin*dIII, subjected to electrophoresis in agarose, and blotted to a nylon membrane. Hybridization to radioactive pMR41 revealed that the two *Hin*dIII fragments of wild-type DNA to which pMR41 hybridized were replaced by a single, larger *Hin*dIII fragment in the *citB84* strain (data not shown).

This putative *citB* mutation was shown to be tightly linked by transformation to *citB75* and *citB1* (Fig. 4). The tight linkage of these mutations suggest that all three reside within the same locus and perhaps the same gene. The wild-type allele of *citB75* appears to lie within the *Hin*dIII to *Eco*RI region of pMR41 (as indicated), and the wild-type allele of *citB1* appears to lie either close to the *Eco*RI site at the end of the cloned DNA within pMR41 or just outside this region (pMR41 does not transform *citB1* to wild type at a detectable frequency). Two caveats regarding the mapping of *citB1*

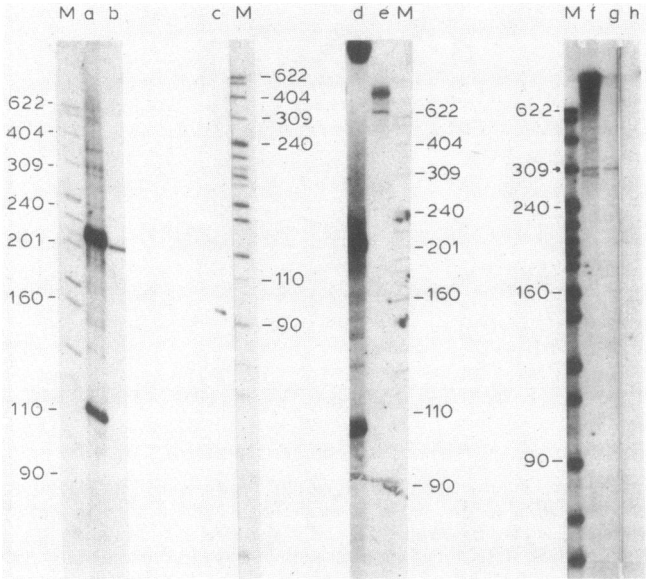

FIG. 8. In vitro transcription of *citB* DNA. Plasmid pMR41 was restricted at various sites in the *citB* region, and run-off transcription was attempted with various forms of *B. subtilis* RNA polymerase. The template restriction site and form of RNA polymerase used for each reaction were as follows: (a) pMR41-*Hin*dIII, Eσ^{55}; (b) S1 mapping with pMR41-*Hin*dIII, transcript II, 200 bases; (c) pMR41-*Pst*I, Eσ^{55}; (d) pMR41-*Hin*dIII, Eσ^{55}; (e) pMR41-*Hin*dIII, Eσ^{29}; (f) pLS5-11ΔRI-*Eco*RI, Eσ^{32} and Eσ^{37}; (g) pLS5-11ΔRI-*Eco*RI, Eσ^{37}; (h) pMR41-*Hin*dIII, Eσ^{37}. pLS5-11ΔRI (M. Fine and A. L. Sonenshein, unpublished data) is a template that contains the *spoVG* promoter located about 300 bp upstream from an *Eco*RI site. Lanes marked M contained marker fragments (pBR322-*Hpa*II). Sizes of fragments are given in bases.

should be mentioned. First, this mutation was isolated in *B. licheniformis* and subsequently transformed into *B. subtilis* (55). Second, *citB1* may contain two mutations affecting aconitase. When *citB1* was transformed by wild-type DNA to glutamate prototrophy, two classes of colonies arose. Large colonies had wild-type levels of aconitase, but the smaller, more frequent class contained only low levels of aconitase. Therefore, the linkage data presented here may represent the location of only one of two mutations carried by strain 1A331.

DISCUSSION

We are studying the *citB* gene of *B. subtilis* to explore the mechanism of catabolite repression in this organism. Aconitase, the apparent product of *citB*, is required for growth on nonfermentable carbon sources and for synthesis of glutamate. Previously, it was demonstrated that aconitase levels are regulated by the combination of the carbon source and a source of glutamate (27, 28, 51). To determine the nature of this regulation, we isolated a 1.1-kb fragment of *B. subtilis* DNA capable of transforming the aconitase-deficient mutant 1A120 (*citB75*) to CitB$^+$. Using this DNA as a hybridization probe, we found that two transcripts (I and II) are initiated in opposite directions within this region from sites which lie about 140 bp apart. Both transcripts are present at higher levels in cells grown in citrate-glutamine medium than in cells grown in glucose-glutamine medium. These are the first demonstrations of transcripts whose levels in vegetative *B. subtilis* are regulated by the carbon source. Nicholson and Chambliss (41) have shown by gene fusion experiments that activity of the α-amylase promoter region is also reduced in the presence of glucose.

Transcript II is regulated synergistically by glucose and glutamine to an extent sufficient to explain the regulation of aconitase activity. This transcript appears to be required for aconitase activity, since a cloned copy of this region which had been mutated in vitro was able to generate CitB$^-$ mutants. Therefore, transcript II appears to be a *citB* transcript and may encode aconitase, a subunit of aconitase, or a factor necessary for synthesis of aconitase. Aconitase consists of a single polypeptide of 79,000 daltons in *S. cerevisiae*, porcine heart, and bovine heart (48), but has not been purified from *B. subtilis* or other bacteria. Transcript II extends at least to within 180 bp of the end of the cloned *citB* region DNA. This fact, along with the inability of this cloned DNA to transform the *citB1* allele, suggests that this DNA does not contain the entire *citB* locus. In fact, the part of transcription unit II that we have cloned could only code for a polypeptide of 20,000 M_w.

A transcript indistinguishable from transcript II in size and location was synthesized in vitro by the major form of RNA polymerase (Eσ^{55}) from vegetative cells. Sequences with good homology to the consensus sequences for promoters recognized by *B. subtilis* Eσ^{55} (39) have been found appropriately positioned upstream of the start site for transcript II (D. Dingman, unpublished data). This promoter region has been fused to the *lacZ* gene of *E. coli*, and the fusion was been introduced into the *B. subtilis* chromosome. Expression of β-galactosidase was observed in log-phase cells but was greatly reduced when glucose rather than citrate was the carbon source (Dingman et al., in preparation). It is noteworthy that transcript II was not synthesized in vitro by any of several alternative forms of RNA polymerase found in vegetative or sporulating cells. This fact suggests that none of these forms of RNA polymerase has as its role the transcription of all catabolite-sensitive genes.

The proximity of the initiaton sites for transcripts I and II and the fact that both transcripts are more abundant in cells grown in citrate-glutamine medium than in cells grown in glucose-glutamine medium raise the possibilities that these genes might be coregulated and that both might be involved in the synthesis of aconitase. However, transcript I does not appear to be required for synthesis of aconitase, since the level of transcript I increases substantially during sporulation at a time when aconitase activity has already reached its peak (Dingman et al., in preparation). The appearance of transcript I during slow growth on a poor carbon source (e.g., citrate) can probably be attributed to expression of some sporulation functions at low levels or in some fraction of the population (though only a small fraction of the population forms heat-resistant spores).

Although regulation of the levels of transcripts I and II appears distinct in several respects, the possibility remains that both transcripts are regulated by the carbon source by partially overlapping mechanisms. For example, the level or activity of a single regulatory protein that binds to the region between the two initiation sites may respond to carbon availability, but other factors, such as binding of different forms of RNA polymerase to the two promoters, may cause these transcripts to be synthesized at different times during the life cycle of the cell.

The identification and isolation of the promoter region for transcript II will allow the first moelcular analysis of regulation in *B. subtilis* by the carbon source and a source of glutamate (and may shed light on the mechanism of regulation of other genes by the carbon source). The only aspect of

aconitase regulation which had been addressed previously was the nature of the intracellular effectors. It is unlikely that glutamine or glutamate are direct effectors, since their pools do not correlate with aconitase specific activity under various growth conditions (15, 17). The 2-ketoglutarate pool, however, is consistently correlated inversely with the level of aconitase and is the most likely candidate for the intracellular effector of regulation by glutamate-related compounds (15, 28, 29). Catabolite regulation of other enzymes such as alpha-glucosidase and inositol dehydrogenase is apparently not affected by the size of the 2-ketoglutarate pool, since the levels of these enzymes remain derepressible in a 2-ketoglutarate dehydrogenase-deficient strain which accumulates large amounts of 2-ketoglutarate (14, 15).

The intracellular mediator(s) of the glucose effect is less well defined. A reduction in the pyruvate pool is associated with relief of catabolite repression of aconitase, histidase, and alpha-glucosidase (13, 15, 17). It is not clear, however, whether these changes in the pyruvate pool are the cause or the result of increased expression of tricarboxylic acid cycle enzymes.

In conclusion, use of the cloned *citB* locus may allow identification of regulatory proteins and sites involved in control of gene expression by the carbon source and a source of glutamate. These identifications would add greatly to our understanding of how the levels of citric acid cycle enzymes in *B. subtilis* respond to both energetic and biosynthetic requirements. The complex relationship between the regulation of *citB* expression and the initiation of sporulation (50) will be addressed in a separate publication (Dingman et al., in preparation).

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