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Relationship between Aconitase Gene Expression and Sporulation in *Bacillus subtilis*

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The *citB* gene of *Bacillus subtilis* codes for aconitase (D. W. Dingman and A. L. Sonenshein, J. Bacteriol. 169:3060–3065). By direct measurements of *citB* mRNA levels and by measurements of β-galactosidase activity in a strain carrying a *citB-lacZ* fusion, we have examined the expression of *citB* during growth and sporulation. When cells were grown in nutrient broth sporulation medium, *citB* mRNA appeared in mid- to- late-exponential phase and disappeared by the second hour of sporulation. This timing corresponded closely to the kinetics of appearance of aconitase enzyme activity. Decoyinine, a compound that induces sporulation in a defined medium, caused a rapid simultaneous increase in aconitase activity and *citB* transcription. After decoyinine addition, the rate of increase in aconitase activity in a 2-ketoglutarate dehydrogenase (*citK*) mutant and in a citrate synthase (*citA*) mutant was significantly less than in an isogenic wild-type strain. This is apparently due to a failure to deplete 2-ketoglutarate and accumulate citrate. These metabolites might act as negative and positive effectors of *citB* expression, respectively. Mutations known to block sporulation at an early stage (*spo0H* and *spo0B*) had no appreciable effect on *citB* expression or aconitase activity. These results suggest that appearance of aconitase is stimulated by conditions that induce sporulation but is independent of certain gene products thought to act at an early stage of sporulation.

Aconitase [EC 4.2.1.3; citrate (isocitrate) hydrolase], a tricarboxylic acid (TCA) cycle enzyme, appears to be subject to at least two forms of regulation in *Bacillus subtilis*. Catabolite repression of aconitase activity occurs whenever a rapidly metabolizable carbon source (e.g., glucose or mannose) is present in the medium (6). Catabolite repression is known to lower overall TCA cycle activity (6) and is thought to be a major regulating force for sporulation (20, 33). For catabolite repression of aconitase to be complete, however, the medium must also contain a good source of 2-ketoglutarate (2-KG) (20, 30). In the absence of a good carbon source, a high intracellular concentration of 2-KG has no detectable effect on aconitase activity (19). Thus, cells growing in a defined medium containing glucose and either glutamine or glutamate have very low aconitase activity, but cells growing in a medium that has citrate as carbon source or ammonia as nitrogen source have high levels of aconitase. No evidence for feedback inhibition of aconitase by any TCA cycle intermediate has been obtained (30).

When cells of *B. subtilis* exhaust a complex medium and begin to sporulate, aconitase activity (which is at a very low level during growth) increases substantially 1 to 3 h after the onset of sporulation (14, 16, 36). This behavior might be taken to mean that aconitase is subject to sporulation control or that the signal that initiates sporulation also activates aconitase expression. Since aconitase activity in this case appears after the end of growth, it is possible that its regulation during sporulation is distinct from its regulation during growth in a defined medium.

Mutants of *B. subtilis* lacking aconitase activity require glutamate (or glutamine) for growth and, after exhaustion of a complex medium, become blocked at stage 0 or I of sporulation (15, 39). This again suggests a linkage between sporulation and aconitase expression. Three closely linked aconitase mutations (*citB1*, *citB75*, and *citB84*) have been isolated and mapped in *B. subtilis* (32, 40). The existence of such mutants allowed us to subclone from a λ gene bank of *B. subtilis* DNA (11) a 1-kilobase fragment that contains part of the *citB* locus (32). The *citB* promoter region has been located on this fragment (32), and the transcriptional and translational start points for aconitase synthesis have been determined (8). The cloned *citB* DNA has been used as a hybridization probe to show that the combined effects of glucose and glutamine in defined medium are to reduce the level of *citB* mRNA (32). This indicates that catabolite repression of aconitase (*citB*) is a transcriptional phenomenon in *B. subtilis*.

In the present work we have sought to clarify the relationship between *citB* mRNA synthesis and sporulation. We found that in complex medium *citB* mRNA appeared in late logarithmic growth phase. Aconitase activity appeared with the same kinetics as did *citB* mRNA, contradicting earlier reports that aconitase appears after the start of stationary phase (14, 16, 36). Experiments that use the compound decoyinine (an inducer of sporulation) showed that induction of *citB* is at the transcriptional level and implicate 2-KG and citrate as metabolite effectors of this induction. Although activation of *citB* expression occurred at the onset of sporulation and aconitase activity is required for sporulation under most conditions, expression of *citB* mRNA was not prevented by two mutations that block sporulation at stage 0. These findings suggest that aconitase expression is induced by the same conditions that induce sporulation, but is not dependent on certain aspects of sporulation-specific regulation.

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TABLE 1. Bacterial strains

Strain	Genotype	Source
E. coli		
MM294	*endA hsdR thi pro*	R. Losick
RV	*Δlac thi*	M. Malamy
B. subtilis		
SMY	Wild type	P. Schaeffer
SMY (pDWD1)	*Φ(citB'-lacZ) kan*	SMY × pDWD1
SmY::pVRD1	*Φ(citB'-lacZ) cat*	SMY × pVRD1
1A120	*citB75 trpC2*	BGSC[a]
LDD-1	*trpC2*	1A120 × DNA of SMY
BCDC-8	*Φ(citB'-lacZ) cat trpC2*	1A120 × DNA of SMY::pVRD1
JH648	*trpC2 pheA1 spo0B136*	J. A. Hoch
ZB480	*chr*::Tn*917*ΩHU146 *trpC2 pheA1 spo0HΔHin*dIII	R. Losick
JH648::pVRD1	*trpC2 pheA1 spo0B136 cat Φ(citB'-lacZ)*	JH648 × DNA of SMY::pVRD1
ZB480::pVRD1	*Φ (citB'-lacZ) cat chr*::Tn*917*ΩHU146 *pheA1 spo0HΔhin*dIII	ZB480 × pVRD1
SF109	*trpC2 glyB4 metC3 citK109*	S. Fisher
SF109S	*glyB4 metC3 citK109*	SF109 × DNA of SMY
BS-8109	*Φ(citB'-lacZ) cat citK109*	BCDC-8 × DNA of SF109S
HS1A17	*trpC2 citA*(Ts)	R. Hanson
BCHS-81A	*Φ(citB'-lacZ) cat citA*	BCDC-8 × DNA of SMY and HS1A17

[a] BGSC, Bacillus Genetic Stock Center, Ohio State University, Columbus.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains used are shown in Table 1. Plasmid pMR41 contains the *citB* promoter region and has been described previously (32). The *lacZ* fusion plasmid pCED6 (9) and pDEB1 (34; D. Bohannon, personal communication) were used as cloning vehicles. Plasmids pDWD1 and pVRD1 were constructed by inserting a 600-base pair *Ava*I-*Hin*dIII fragment of *B. subtilis* DNA from pMR41 into the *Hin*dIII sites of pCDE6 and pDEB1, respectively (Fig. 1). (The *Ava*I end of the insert was first changed to a *Hin*dIII site by cutting pMR41 with *Ava*I, filling in the ends by use of the large [Klenow] fragment of DNA polymerase, and adding *Hin*dIII linkers by blunt-end ligation.) The orientation of the insert in each plasmid was shown by restriction mapping to be such that the *citB* promoter was proximal to and directed toward *lacZ*.

Culture media. *Escherichia coli* strains were grown in L broth or on L-agar plates (27). When appropriate, the growth medium contained 10 μg of ampicillin (Amp) per ml and 5 μg of chloramphenicol (Cam) or kanamycin (Kan) per ml. *B. subtilis* strains were grown in DSM [0.8% nutrient broth, 0.1% KCl, 0.025% $MgSO_4 \cdot 7H_2O$, 1.0 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2$, 1.0 μM $FeSO_4$ (35)] or TSS (0.05 M Tris, pH 7.5, 40 μg of $FeCl_3 \cdot 6H_2O$-Na_3 citrate per ml, 2.5 mM K_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% NH_4Cl, 0.5% glucose [12]) liquid medium. For plates of DSM and TSS media, agar was added to 17 g/liter. When necessary, TSS medium was supplemented with amino acids (0.004%). Unless indicated otherwise, the antibiotic Cam or Kan was added to a final concentration of 5 μg/ml when appropriate. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was added to solid media (40 μg/ml) as an indicator of β-galactosidase activity.

DNA manipulations. Methods for endonuclease digestion and DNA ligation were as described by Maniatis et al. (26). Chromosomal DNA was isolated from *B. subtilis* by the procedure of Sonenshein et al. (35). Plasmid DNA was isolated from *E. coli* by procedures appropriate to small-scale (22) or large-scale (3) preparations. Restriction enzymes, T4 DNA ligase, DNA polymerase (Klenow fragment), and *Hin*dIII linkers were obtained from New England BioLabs, Inc. Agarose and native or denaturing (8 M urea) polyacrylamide gels were prepared and electrophoresed in the buffers described by Maniatis et al. (26).

Transformation. *E. coli* and *B. subtilis* strains were transformed by the competent cell techniques of Davis et al. (7)

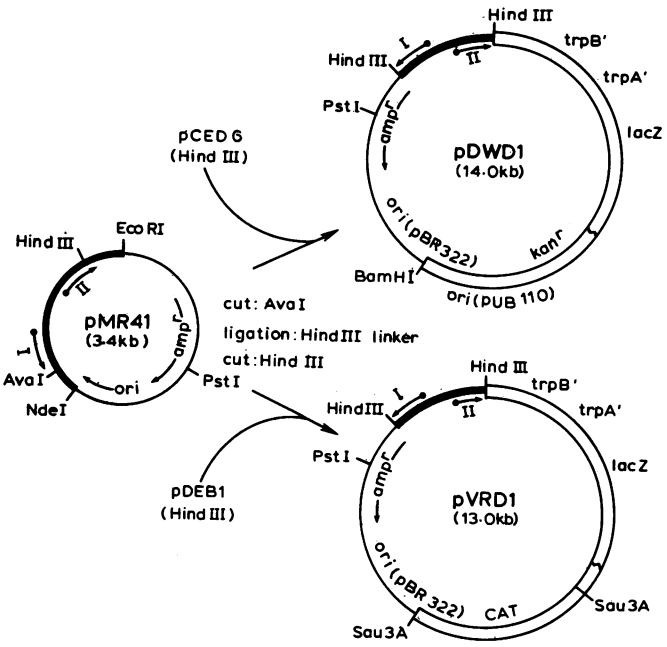

FIG. 1. Construction of the *citB-lacZ* fusion plasmids pDWD1 and pVRD1. For details of construction, see Materials and Methods. The arrow labeled II indicates the origin and orientation of *citB* mRNA; the arrow labeled I indicates an upstream, divergent transcript. kb, Kilobases.

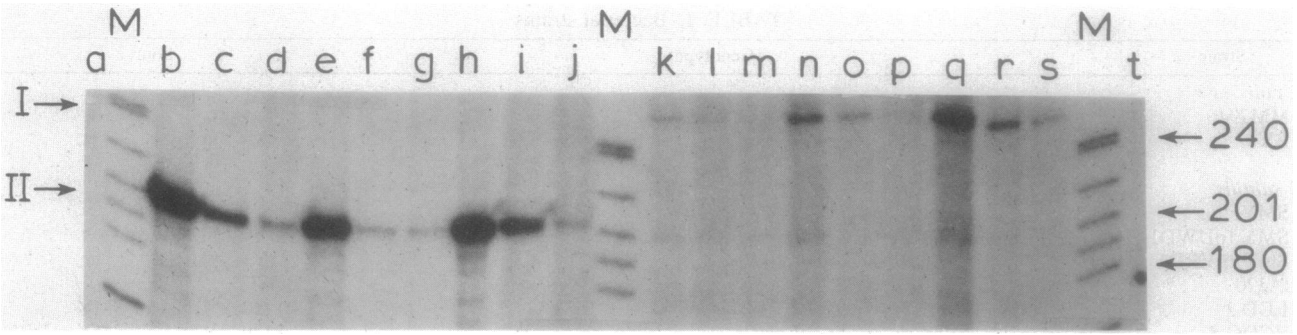

FIG. 2. Levels of *citB* and transcript I mRNA during growth and sporulation in DSM medium. Threefold dilutions of RNA (all brought to 30 μg with *Saccharomyces cerevisiae* RNA) were hybridized to 5′-end-labeled DNA of a mixture of pMR41 cleaved with *Aval* (from which transcript I protects a fragment of 270 bases) and pMR41 cleaved with *Hin*dIII (from which *citB* mRNA [transcript II] protects a fragment of 200 bases). Mixing the two probes allowed each to serve as an internal control for the other. Hybridized samples were treated with S1 nuclease, denatured, and electrophoresed in 8 M urea–6% polyacrylamide gels. Lanes are as follows: pBR322/*Hpa*II size markers (M); *S. cerevisiae* RNA (a, t); 30, 10, 3.3 μg of vegetative (T_{-1}) RNA (b, c, d); 30, 10, 3.3 μg of T_0 RNA (e, f, g); 30, 10, 3.3 μg of T_1 RNA (h, i, j); 30, 10, 3.3 μg of T_2 RNA (k, l, m); 3, 1, 0.33 μg of T_3 RNA (n, o, p); and 3, 1, 0.33 μg of T_4 RNA (q, r, s).

and Dubnau and Davidoff-Abelson (10), respectively. *E. coli* transformants were selected on L agar containing X-gal, Amp, and Cam or Kan. *B. subtilis* transformants were selected by plating on L plates containing X-gal and Cam or Kan or on TSS plates containing Cam, Kan, or the appropriate amino acids. Screening for TCA cycle mutant transformants of *B. subtilis* was by the method of Carls and Hanson (4).

Enzyme assays. For β-galactosidase assays, cells were harvested by centrifugation (12,000 × *g*, 5 min), washed with 50 mM Tris hydrochloride (pH 8.0; 4°C), and frozen overnight at −20°C. Thawed cells were made permeable by suspension in a freshly prepared emulsion of 50 mM Tris hydrochloride (pH 8.0) plus 0.1% toluene. β-Galactosidase activity was measured by a modification of the *o*-nitrophenylgalactoside hydrolysis procedure described by Miller (27). Before measurements of A_{420}, the reaction mixture was centrifuged to remove any turbidity due to the permeabilized cells.

Preparation of cleared cellular extracts and both activated and unactivated assays for aconitase were as described previously (8). Protein concentrations were determined by the method of Lowry et al. (25), using bovine serum albumin as the standard.

Mapping transcripts with S1 nuclease. Conditions for end labeling of DNA, hybridization, and S1 nuclease treatment have been described previously (32).

Induction by decoyinine. Decoyinine U-7984 (kindly provided by G. B. Whitfield and R. L. Keene, The Upjohn Co.) was used according to the procedure of Uratani-Wong et al. (37). Starter cultures of *B. subtilis* were grown in 5 ml of TSS medium containing glucose (0.5%) and glutamine (0.2%) at 37°C for approximately 3 h. These cultures were then diluted to 60 ml with fresh medium and incubation was continued as before. Growth of the cultures was monitored with a Klett-Summerson photocolorimeter, using a green (540-nm) filter. When the turbidity of a culture reached 100 Klett units, it was divided into two 20-ml portions. To one portion was added 0.1 ml of 1 M KOH (control culture). To the other portion was added 0.1 ml of decoyinine (100 mg/ml, dissolved in 1 M KOH). The final decoyinine concentration in this culture was 1.8 mM. Both portions were then incubated as before. At selected time points before and after addition of decoyinine, samples (5 ml) were removed and stored for aconitase and β-galactosidase assays (see above).

RESULTS

Appearance of *citB* mRNA during growth and sporulation. In previous experiments we showed that the steady-state level of *citB* mRNA during growth in minimal medium varies with the carbon source (32). That is, cells grown in glucose-glutamine medium have 10- to 20-fold less *citB* mRNA than do cells grown in citrate-glutamine or glucose-ammonia medium. Since aconitase, the product of *citB* (8), had been shown to have a maximum specific activity between the first and second hours of sporulation (14, 16, 36; M. S. Rosenkrantz, Ph.D. thesis, Tufts University, Boston, Mass., 1984), we sought to determine when, during sporulation, transcription of *citB* occurs.

B. subtilis SMY was grown in nutrient broth sporulation medium (DSM), and RNA was isolated from cells harvested during late exponential growth and at several times during sporulation. This RNA was probed for transcripts from the *citB* region, using the S1 mapping procedure of Berk and Sharp (2).

We had previously shown that the *citB* transcript protects a 200-base fragment of plasmid pMR41 that had been cleaved at its *Hin*dIII site and labeled at its 5′ end. Surprisingly, this transcript was present during late exponential growth but had disappeared by the second hour of sporulation (Fig. 2). This implies that aconitase specific activity reaches a peak at a time at which *citB* mRNA is no longer present in the cells.

The *citB* region contains a second transcript (referred to as transcript I in reference 32). Its start point is located 140 base pairs upstream of the *citB* start point, and it is synthesized in the opposite direction from *citB* mRNA. Transcript I (detected as a 270-base protected fragment) is a sporulation-specific mRNA (Fig. 2). It begins to appear 2 h after the beginning of sporulation and accumulates thereafter. Since this transcript appears after aconitase specific activity reaches its peak, it is unlikely that it is responsible for aconitase expression.

Our results suggest that neither of two transcripts from the *citB* region appears at a time consistent with the previously reported appearance of aconitase activity. This raised the possibilities that *citB* gene expression was not the limiting factor for appearance of aconitase activity or that previous measurements of the appearance of aconitase activity were misleading.

Fusion of the *citB* promoter to *lacZ*. To investigate further

the discrepancy between the appearance of *citB* mRNA and aconitase activity and to monitor transcription of *citB* more easily, we fused the *citB* promoter region to the structural gene for *E. coli* β-galactosidase (*lacZ*), using plasmids pCED6 (Kan^r) and pDEB1 (Cam^r) (see Materials and Methods). Both plasmids contain a unique *Hin*dIII site preceding a promoterless *lacZ* gene. The ribosome-binding site for this *lacZ* gene is active in both *B. subtilis* and *E. coli* (9). Insertion of the 600-base pair *Ava*I-*Hin*dIII fragment from pMR41 at the *Hin*dIII site created plasmids pDWD1 and pVRD1, which are derivatives of pCED6 and pDEB1, respectively (Fig. 1). pDWD1 replicates autonomously in *B. subtilis*, under the control of its pUB110 replicon, and is present in multiple copies. pVRD1 contains no replicon functional in *B. subtilis*; transformants to Cam^r arise by Campbell-type insertion of the plasmid at the *citB* promoter site of the chromosome. This creates a strain with a low number of copies of the *citB-lacZ* fusion (in principle, a single copy per chromosome).

Plasmid pVRD1 was transformed into *B. subtilis* SMY, and chromosomal DNA from this clone (SMY::pVRD1) was used to transform *B. subtilis* 1A120 (*citB75 trpC2*) to Cam^r. Of 108 Cam^r transformants, 36% became Cit$^+$, indicating close linkage of the *citB* locus and the *citB-lacZ* fusion. One such transformant strain was called BCDC-8 [ϕ(*citB'-lacZ*) *cat trpC2*].

Table 2 shows the specific activities of β-galactosidase and aconitase in strains which have these *citB-lacZ* fusions and in their parent strains. The specific activity levels of aconitase in strain BCDC-8 were comparable to those measured in strain SMY under both repressing and derepressing conditions. Under repressing conditions, strain LDD-1 (a Cit$^+$ derivative of strain 1A120, the parent of strain BCDC-8) had a specific activity of aconitase comparable to that of strain BCDC-8. This indicates that the presence of the *citB-lacZ* fusion in strain BCDC-8 has no significant effect on catabolite regulation of aconitase. The β-galactosidase activity (*citB-lacZ* expression) in this strain was also subject to catabolite regulation, confirming that the effect of glucose on the *citB* gene is transcriptional. As expected, the strain with the multicopy fusion had higher β-galactosidase levels than did the strain in which the fusion was integrated into the chromosome. Strain SMY(pDWD1), in fact, had very high β-galactosidase activities under either repressing or derepressing conditions. Due to plasmid instability in this strain, it is difficult to know for certain whether β-galactosidase activity is at all under catabolite regulation. The specific activity of aconitase in this strain did show some regulation by the carbon source, but much less than that observed for its plasmidless parent. Under repressing conditions, the level of aconitase activity was higher in strain SMY(pDWD1) than in strain SMY. This would be consistent with partial titration of a negative regulator by the multicopy plasmid.

Activity profiles of aconitase and β-galactosidase. To compare directly aconitase activity levels and transcription of *citB* (as deduced from β-galactosidase activity), strains SMY and BCDC-8 were grown in DSM medium, and at various times during growth and sporulation samples were withdrawn and assayed for aconitase or β-galactosidase activities or both. Figure 3A shows that the kinetics of appearance of aconitase activity in the wild-type strain (SMY) varied according to the protocol used to assay the enzyme. Assays by the standard protocol showed the enzyme beginning to appear at the end of growth and reaching a peak between T_0 and T_1. This is in accord with previous results (14, 16, 36).

However, when the extracts of *B. subtilis* SMY cited above were exposed to an activating mixture (8), aconitase activity could be seen to appear well before T_0 (Fig. 3A). When extracts of strain BCDC-8 were assayed for β-galactosidase and aconitase (after activation), the two enzymatic activities had the same kinetics of appearance (Fig. 3B). Since the pH of the medium rose (indicative of TCA cycle activity) at the same time as did the two enzyme activities (Fig. 3B), we conclude that *citB* gene expression and aconitase activity in vivo increase concurrently in mid- to late exponential phase and not after T_0.

Thus, aconitase is made during vegetative growth in complex medium, but only after some components of the medium have been metabolized. This is consistent with the high activity of aconitase in exponentially growing cells in a minimal medium containing a poorly metabolized carbon source (e.g., citrate) and the low activity of aconitase in the same medium supplemented with glucose.

Induction of aconitase activity by decoyinine. Freese and colleagues (28) have reported that the addition of decoyinine to a culture of exponentially growing *B. subtilis* induces sporulation in a minimal medium. This compound inhibits GMP synthetase, resulting in a drop in GDP and GTP pools. A decrease in the GTP pool is characteristic of all conditions of sporulation induction, but the mechanistic relationship between the size of the GTP pool and sporulation induction is unknown. Aconitase activity has been shown to increase after addition of decoyinine (37). This is true even in a medium containing excess glucose and glutamate, implying that decoyinine can override catabolite repression of aconitase. However, it was not known whether decoyinine induces aconitase activity by stimulating *citB* expression.

Decoyinine is only effective in a medium lacking purines (28). We have found that TSS medium (a minimal medium) is suitable for decoyinine induction of sporulation. Strain BCDC-8 was grown in TSS medium containing glucose, glutamine, Cam, and tryptophan. Decoyinine was added as described in Materials and Methods. Figure 4 shows that, as has been previously reported for other strains (37), decoyinine stimulates aconitase activity in BCDC-8. The increase in activity occurred within 30 min after decoyinine addition. The activity of β-galactosidase was also induced by

TABLE 2. Specific activities of β-galactosidase and aconitase in strains with the *citB-lacZ* fusion[a]

Strain	Medium[b]	Sp act (U/mg of protein)	
		β-Galactosidase	Aconitase[c]
SMY	TSS (glu; gln)	<3	15.6
	TSS (cit; gln)	11.7	165.0
SMY(pDWD1)[d]	TSS (glu; gln)	2,250	36.0
	TSS (cit; gln)	2,890	93.3
1A120[e]	TSS (glu; gln)	<3	<3
	TSS (cit; gln)	ND[f]	ND
LDD-1	TSS (glu; gln)	<3	15.8
	TSS (cit; gln)	ND	ND
BCDC-8[g]	TSS (glu; gln)	11.3	20.7
	TSS (cit; gln)	290.0	112.0

[a] All strains were grown at 37°C and harvested when growth reached 100 Klett units.
[b] Medium was supplemented with glucose (glu) and glutamine (gln) or citrate (cit) and glutamine.
[c] Measured by the activated aconitase procedure.
[d] Growth medium contained Kan (1 μg/ml).
[e] Growth medium contained tryptophan (0.004%).
[f] ND, Not determined.
[g] Growth medium contained Cam (1 μg/ml) and tryptophan (0.004%).

the addition of decoyinine. This increase in expression of *citB* occurred 15 to 30 min after decoyinine addition. This result indicates that the appearance of aconitase after induction of sporulation by decoyinine is due to activation of transcription of the *citB* gene.

Effect of decoyinine on *citB* transcription in a 2-KDH mutant. That there was a 15- to 30-min lag in *citB* expression after decoyinine addition suggests that the effect of decoyinine is indirect. Decoyinine is known to cause an increase in the specific activity of 2-ketoglutarate dehydrogenase (2-KDH) (37). As expected, this results in a drop in the intracellular pool of 2-KG. Since 2-KG is thought to be involved in negative regulation of *citB* expression (12, 30), the lag between the time of decoyinine addition and expression of *citB* might reflect the time needed to induce 2-KDH and deplete 2-KG. To test this possibility, decoyinine induction of aconitase activity was measured in a 2-KDH (*citK*) mutant.

Strain BS-8109 is a *citK* mutant derived by transforming strain BCDC-8 with DNA from strain SF109. The *citK* mutation causes an increase in the 2-KG pool in cells grown in TSS medium (12). Figure 5A shows that in strain BS-8109 induction of *citB* transcription was significantly lower than in control strain BCDC-8. This result supports the idea that depletion of the 2-KG pool is required for full *citB* induction by decoyinine. That *citB* induction in strain BS-8109 was not completely prevented suggests that 2-KG is not the only metabolite that regulates *citB*.

Effect of decoyinine on *citB* transcription in a citrate synthase mutant. Decoyinine is known to induce citrate synthase as well as aconitase and 2-KDH (37). Since citrate has been proposed as an inducer of aconitase (30), we sought to determine whether accumulation of citrate plays a role in induction of *citB* by decoyinine.

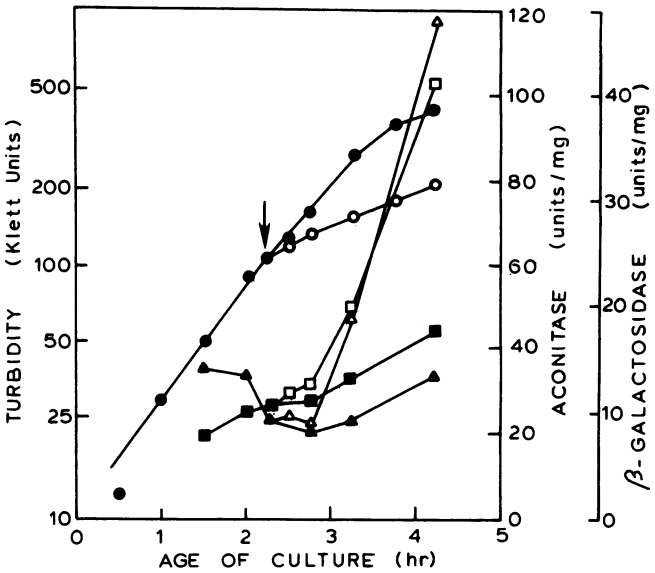

FIG. 4. Induction of aconitase activity and *citB* expression by decoyinine. *B. subtilis* BCDC-8 was grown at 37°C in TSS medium supplemented with glucose, glutamine, Cam (5 μg/ml), and tryptophan (0.004%). Samples were removed periodically and assayed for turbidity, aconitase activity, and β-galactosidase activity. When the turbidity reached 100 Klett units, the culture was split into two 20-ml portions to which 0.1 ml of either 1 M KOH (control) or decoyinine (100 mg/ml in 1 M KOH) was added. Symbols: (○, ●) turbidity; (△, ▲) aconitase activity; (□, ■) β-galactosidase activity. Open symbols represent the decoyinine-treated culture; closed symbols are for the control culture.

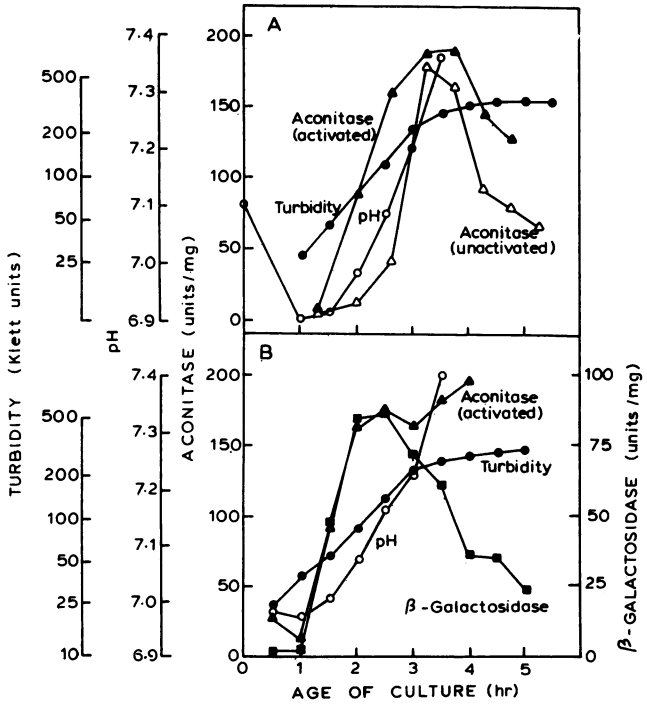

FIG. 3. Activity profiles of aconitase and β-galactosidase in *B. subtilis* SMY (A) and BCDC-8 (*citB-lacZ* fusion) (B). Both strains were grown in DSM medium (the medium for strain BCDC-8 also contained Cam [5 μg/ml]) at 37°C, and samples were assayed periodically for turbidity (●), pH of the medium (○), aconitase activity (activated [▲] and standard procedure [△]), and β-galactosidase activity (■).

An appropriate strain carrying a citrate synthase (*citA*) mutation was created by transforming strain BCDC-8 (*trpC2 citA*$^+$) simultaneously with saturating amounts of DNA from strains SMY (wild type) and HS1A17 (*trpC2 citA1*). Trp$^+$ transformants were selected and screened for acquisition of the Cit$^-$ phenotype by congression. A resulting strain, BCHS-81A, was found to lack citrate synthase activity. Figure 5B shows that transcription of *citB* in strain BCHS-81A was not induced by decoyinine. This finding shows that citrate is required for *citB* expression.

Thus, activation of aconitase gene expression during induction of sporulation by decoyinine requires depletion of 2-KG and accumulation of citrate.

Aconitase expression in *spo0B* and *spo0H* mutants. Although expression of *citB* in cells in nutrient broth medium occurs prior to the end of growth, it is not necessarily unrelated to sporulation. A similar situation obtains for the *spoVG* gene (41). To relate expression of *citB* to the beginning of sporulation, we tested *citB* transcription in two mutants known to be blocked at the onset of sporulation.

Strains JH648 (*spo0B136*) and ZB480 (*spo0HΔHin*dIII) were transformed to Cam^r by using DNA from strain SMY::pVRD1 and plasmid pVRD1, respectively. The transformants, JH648::pvRD1 and ZB480::pVRD1, were grown in DSM medium containing Cam (5 μg/ml). Samples were removed periodically for assays of aconitase and β-galactosidase. Figure 6 shows that neither the *spo0B* mutation nor the *spo0H* mutation had any appreciable effect on *citB* expression or aconitase activity. Strains carrying

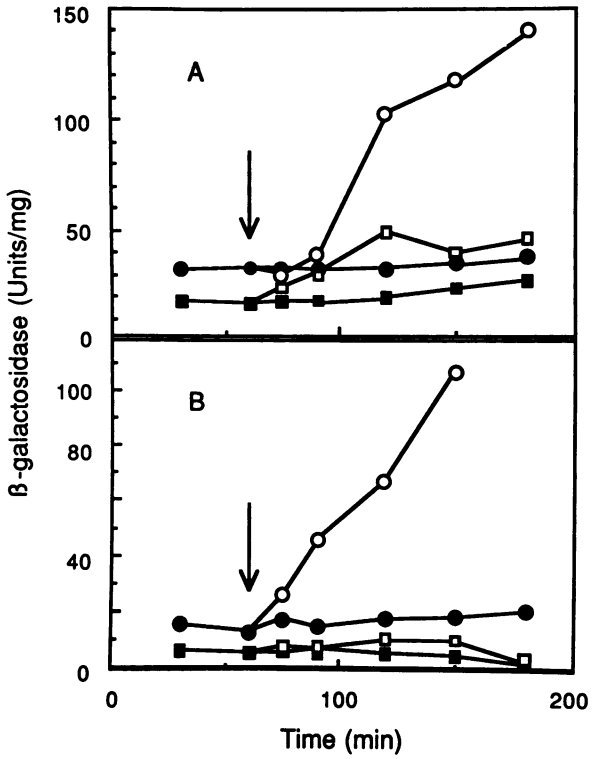

FIG. 5. Induction of *citB* expression in *citK* (A) and *citA* (B) mutants by decoyinine. *B. subtilis* BS-109, BCHS-81A, and BCDC-8 were grown at 37°C in TSS medium containing glucose, glutamine, Cam (5 μg/ml), and tryptophan (0.004%). Samples were taken periodically and assayed for β-galactosidase activity. At the time point indicated by the arrows (100 Klett units), each culture was split into two 20-ml portions. To each portion was added 0.1 ml of either 1 M KOH (control) or decoyinine (100 mg/ml in 1 M KOH). Symbols: (A) (○, ●) BCDC-8; (□, ■) BS-109. (B) (○, ●) BCDC-8; (□, ■) BCHC-81A. Open symbols represent the decoyinine-treated culture; closed symbols represent the control culture.

either of the mutations *spo0H17* or *spo0A3* were also unaffected in *citB* expression (data not shown). This implies that *citB* expression is independent of the functions of the wild-type *spo0A*, *spo0B*, and *spo0H* genes. This behavior distinguishes the *citB* gene from *spoVG* and other genes whose expression is dependent on *spo0* gene functions.

This result can be interpreted to mean that aconitase, while essential for sporulation under some conditions (15, 39), is not a sporulation-specific function. Alternatively, the appearance of aconitase may be dependent on the same signals that initiate sporulation but may occur before the time at which even very early sporulation-specific genes function.

DISCUSSION

During growth and sporulation of *B. subtilis* in a complex medium, expression of the aconitase gene occurs considerably earlier than anticipated or previously reported. Expression begins near mid-exponential growth and reaches a peak in late-exponential growth. Since the onset of sporulation is traditionally (and arbitrarily) defined as the start of stationary phase, this raises the question as to whether *citB* is a sporulation gene. Previous results showing that the *citB* gene is transcribed by the major vegetative form of RNA polymerase ($E\sigma^{43}$) (32) and our finding here that aconitase (*citB*) expression is unaffected by mutations in two regulatory genes for sporulation, which function at the earliest time point in sporulation, suggest that appearance of aconitase activity is not a sporulation-specific event. Still, an active TCA cycle is necessary for sporulation to occur, at least when sporulation is induced by nutrient depletion. Mutations in *citB* block sporulation in this case at stage 0 or I.

Exactly how regulation of aconitase relates to initiation of sporulation is uncertain. All previous data, including our own, show that the same conditions which induce sporulation (i.e., carbon or nitrogen or phosphorous source depletion [20] or addition of decoyinine) also induce aconitase (*citB*). Induction of aconitase by decoyinine appears to be part of a cascade. Uratani-Wong et al. (37) have observed that addition of decoyinine causes a rapid increase in 2-KDH activity (within 5 to 10 min) and a subsequent decrease in the pool of 2-KG (within 10–15 min). It is known that 2-KG levels are inversely correlated with aconitase specific activity during growth (12, 30). The 15- to 30-min delay in appearance of aconitase activity and *citB* mRNA after decoyinine addition may be indicative of this indirect mechanism of induction. The lowered induction of *citB* expression in a 2-KDH mutant adds support to the idea that 2-KG is a regulatory metabolite for the *citB* gene. 2-KG may also contribute to regulation of histidase expression, since histidase specific activity has an inverse correlation with 2-KG pool size (12) and histidase can be induced by decoyinine (S. H. Fisher, personal communication).

Since the 2-KDH mutant does express *citB* to some degree after decoyinine induction, this cascade mechanism may not be the only factor regulating aconitase. Lack of expression of *citB* in a citrate synthase (*citA*) mutant following addition

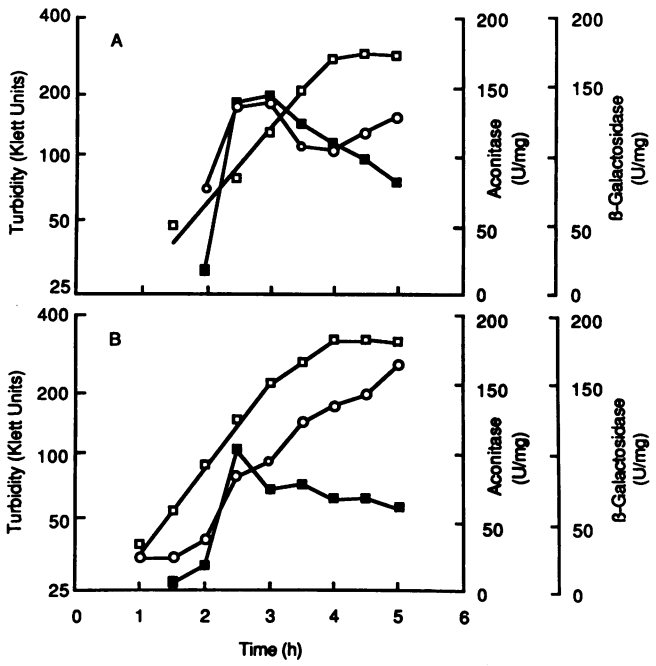

FIG. 6. Activity profiles of aconitase and β-galactosidase in *B. subtilis* JH648::pVRD1 (*spo0B*) (A) and ZB480::pVRD1 (*spo0HΔHind*III) (B). Both strains were grown in DSM medium containing Cam (5 μg/ml) at 37°C, and samples were removed periodically for assay of turbidity, aconitase activity, and β-galactosidase activity. Symbols: (□) turbidity; (○) aconitase activity; (■) β-galactosidase activity.

of decoyinine shows that induction of *citB* by decoyinine also requires synthesis of citrate (Fig. 5B). The complete absence of induction, in fact, may mean that citrate accumulation is an absolute requirement for induction of aconitase.

Thus, aconitase expression may respond to counterbalancing effects of the intracellular levels of citrate and 2-KG. Other factors which alter the activity of citrate synthase and 2-KDH can be viewed as indirect regulators of aconitase expression. For instance, acetate (or acetyl-coenzyme A), which has been implicated as an inducer of 2-KDH (1, 37), increases shortly after addition of decoyinine (37).

Since decoyinine induces both citrate synthase and 2-KDH activities, one might speculate that modulation of the GTP pool by decoyinine causes activation or induction of these enzymes and thereby induces both aconitase and sporulation. If depletion of 2-KG is, in fact, part of the primary pathway by which decoyinine induces sporulation, then *citB* may be a useful paradigm for the earliest expressed sporulation genes. Depletion of 2-KG is probably not essential for sporulation, however, since mutants deficient in 2-KDH can be induced to sporulate by addition of decoyinine (17; data not shown).

Other genes expressed at the onset of sporulation seem to be regulated by mechanisms that partially overlap with that for *citB*. Expression of *spoVG*, for instance, is induced at the end of logarithmic growth and is repressed by glucose (23, 31). This repression can be overcome by decoyinine, allowing *spoVG* expression in the presence of excess glucose and other nutrients (P. Zuber, personal communication). In this sense, regulation of *spoVG* and that of *citB* are very similar. It would be interesting to know whether 2-KDH plays any role in the induction of *spoVG* by decoyinine. The *spoVG* gene differs from *citB* in that it is transcribed by minor forms of RNA polymerase (24) and depends for its expression on the products of several *spo0* genes (31).

The *amyE* gene, although not essential for sporulation, is also induced at the start of stationary phase and its expression is repressed by glucose (5, 21, 29). It is transcribed by the major vegetative form of RNA polymerase and its appearance is not affected by *spo0* mutations (W. Nicholson and G. Chambliss, personal communication).

The *ctc* gene appears to be regulated by the same conditions that govern *citB*, *amyE*, and *spoVG*, but in an inverse way. Although expression of *ctc* occurs at the start of stationary phase, its induction is stimulated in the presence of catabolic repressors (i.e., glucose and glutamine) (23, 31). *ctc* expression is also stimulated in TCA cycle mutants and mutants blocked early in sporulation (23). The *ctc* gene is transcribed by minor forms of RNA polymerase (18, 23).

In the accompanying paper (8), we show that the promoter region of the *citB* gene has substantial sequence homology with the promoters for *spoVG* and *amyE* (24, 38). An economical model would suggest that these sequences reflect the binding sites for proteins that are responsible for the common aspects of regulation of these genes (repression by glucose, dependence on growth phase). Superimposed on these common aspects might be other levels of regulation that involve specific forms of RNA polymerase and specific regulators (*spo0* gene products for *spoVG*, a citrate-sensitive repressor of *citB*).

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