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Regulation of Aconitase Synthesis in *Bacillus subtilis*: Induction, Feedback Repression, and Catabolite Repression

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The synthesis of aconitase in *Bacillus subtilis* wild-type and different citric acid cycle mutants has been studied and the influence of various growth conditions examined. Aconitase is induced by citrate and precursors of citrate and repressed by glutamate. Induction and repression counteract each other, and at equimolar concentrations of citrate and glutamate, aconitase synthesis is unaffected. Induction by citrate can partly overcome catabolite repression of aconitase. Isocitrate dehydrogenase mutants show endogenous induction of aconitase due to citrate accumulation. Leaky mutants defective in citrate synthase and aconitase cannot be induced by citrate, which indicates that they carry a regulatory mutation. The complex regulation of aconitase is discussed with reference to the participation of this enzyme in glutamate biosynthesis and energy metabolism.

The first three enzymes of the citric acid cycle, citrate synthase, aconitase, and isocitrate dehydrogenase, catalyze the condensation of oxalacetate and acetyl coenzyme A to citrate and the isomerization and oxidation of citrate to α-ketoglutarate. The formation and subsequent oxidation of α-ketoglutarate is necessary to regenerate oxalacetate and to keep the citric acid cycle operating. α-Ketoglutarate is also a precursor of glutamate and amino acids derived from glutamate.

In *Bacillus subtilis*, the synthesis of citrate synthase and aconitase appears to be regulated by feedback repression. The addition of glutamate to the growth medium leads to repression of both enzymes (7, 9), and a coordinate regulation of citrate synthase and aconitase has been suggested (7). The results obtained so far have not permitted a definite conclusion as to the nature of the co-repressor(s) involved. It has been shown (16) that not only glutamate, but also α-ketoglutarate and amino acids capable of being converted to glutamate, cause repression of aconitase. The synthesis of isocitrate dehydrogenase appears to be repressed by glutamate at least in some strains of *B. subtilis* (9, 17).

Mutants defective in citrate synthase, aconitase, or isocitrate dehydrogenase require exogenous glutamate for growth (5, 10). A block in aconitase increases the level of isocitrate dehydrogenase and vice versa (5, 28). This has been interpreted to mean that aconitase and isocitrate dehydrogenase are derepressed under conditions of glutamate limitation. Mutants blocked in α-ketoglutarate dehydrogenase, succinate dehydrogenase, or fumarase show repressed levels of aconitase (5, 9, 25, 28). The reason for this pleiotropic phenotype is not known, but it has been suggested that the intracellular concentrations of α-ketoglutarate and glutamate may be elevated in all mutants blocked in the reductive branch of the cycle (25).

The present study deals with the synthesis of aconitase in *B. subtilis* wild-type and various citric acid cycle mutants under different growth conditions. The results show that aconitase is regulated in a complex manner consistent with the participation of this enzyme in both biosynthesis and energy metabolism.

MATERIALS AND METHODS

Bacteria. The *B. subtilis* strains studied are derivatives of strain 168. The mutant strains are listed in Table 1. The isolation and characterization of these mutants has been reported (5, 28). Strain HS1A15 was originally described as defective in both aconitase and isocitrate dehydrogenase. In my hands, this mutant was defective only in isocitrate dehydrogenase. A likely explanation is that the original strain carried two mutations, one of which later reverted.

Media. The minimal glucose medium contained per liter: $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 14 g; KH_2PO_4, 6 g; $MgSO_4(7H_2O)$, 0.2 g; and glucose, 5 g (29). Nutrient sporulation (NSMP) medium was prepared as described by Fortnagel and Freese (10). This medium contained 8 g of nutrient broth (Difco) per liter plus

TABLE 1. *Mutant strains*

Strain	Enzymatic defect	Phenotype symbol	Source
HS1A14	Citrate synthase, aconitase	Cts⁻ Aco⁻	R. S. Hanson
HS2A1	Citrate synthase, aconitase	Cts⁻ Aco⁻	R. S. Hanson
HS3A20	Aconitase	Aco⁻	R. S. Hanson
CitB25	Aconitase	Aco⁻	This laboratory
CitC1	Isocitrate dehydrogenase	Idh⁻	This laboratory
CitC3	Isocitrate dehydrogenase	Idh⁻	This laboratory
CitC6	Isocitrate dehydrogenase	Idh⁻	This laboratory
HS1A15	Isocitrate dehydrogenase	Idh⁻	R. S. Hanson
HS2A2	Isocitrate dehydrogenase	Idh⁻	R. S. Hanson
CitD29	α-Ketoglutarate dehydrogenase	Kdh⁻	This laboratory
CitK14	α-Ketoglutarate dehydrogenase	Kdh⁻	This laboratory
CitF8	Succinate dehydrogenase	Sdh⁻	This laboratory
CitG4	Fumarase	Fum⁻	This laboratory

0.001 mM $FeCl_3$, 0.7 mM $CaCl_2$, 0.05 mM $MnCl_2$, 1.0 mM $MgCl_2$, and 0.1 M potassium phosphate buffer, pH 6.5. Solutions of citric acid cycle intermediates and amino acids were adjusted to pH 6.5, filter sterilized, and added separately to the media.

Purification agar (PA) plates were made from the pH indicator medium containing bromocresol purple described by Carls and Hanson (5). Wild-type colonies are blue on PA plates, whereas acid-accumulating mutants form yellow colonies. Minimal glucose plates were made from minimal glucose medium plus 0.01 mM manganese chloride, 1 g of citrate, and 18 g of agar per liter.

Growth conditions. Bacteria were grown in 50 ml of medium in 250-ml Ehrlenmeyer flasks or in 500 ml of medium in 3-liter Fernbach flasks, Cultures were incubated at 37 C on a rotary shaker at 200 rotations/min. Growth was followed by measuring the absorbance at 600 nm (A_{600}).

Cultures in minimal glucose medium were inoculated with cells from overnight cultures in the same medium. Cultures in NSMP medium were inoculated with cells from overnight PA plates. The purity of mutant cultures was routinely checked by spreading samples on PA plates at the end of each experiment.

Preparation of extracts for enzyme assays. Crude extracts were prepared as described earlier (25) except that the buffer contained 50 μg of chloramphenicol per ml. All enzyme assays were performed within 2 h after the preparation of extracts because of instability of aconitase and fumarase.

Protein was determined according to Lowry et al. (22) with crystalline bovine serum albumin as standard.

Enzyme assays. The enzyme reactions were followed in a Hitachi 101 spectrophotometer. The assays were carried out at room temperature. Specific activities were calculated as nanomoles of substrate converted per minute per milligram of protein.

Aconitase (EC 4.2.1.3, citrate [isocitrate]hydrolyase) was assayed by the method of Racker (26). The reaction mixture contained 10 mM DL-isocitrate in 50 mM potassium phosphate buffer, pH 7.4. The conversion of isocitrate to *cis*-aconitate was followed at 240 nm, and the molar extinction coefficient for *cis*-aconitate was taken to be 3.3×10^3 cm^{-1} M^{-1}.

Isocitrate dehydrogenase (EC 1.1.1.42, threo-D_s-isocitrate: nicotinamide adenine dinucleotide phosphate (NADP) oxidoreductase [decarboxylating]) (reference 20) and fumarase (EC 4.2.1.2, L-malate hydro-lyase) (reference 16) were measured as described in the references. The molar extinction coefficients for reduced NADP and fumarate were taken to be 6.22×10^3 cm^{-1} M^{-1} (reference 18) and 2.4×10^3 cm^{-1} M^{-1} (reference 3), respectively.

Genetic analysis. Transforming deoxyribonucleic acid (DNA) was prepared by a modification of the Marmur method (27). DNA concentrations were determined by the method of Burton (4) with calf thymus DNA as standard. The method for transformation was described by Anagnostopoulos and Spizizen (1). DNA was added to a final concentration of 0.05 μg/ml.

The recombination index method (2) was used to show linkage of *cit* markers. DNA from a *cit trp*⁺ strain and from the wild strain was used to transform a *cit trpC2* strain for *cit*⁺ and *trp*⁺. The mutants studied, except the isocitrate dehydrogenase mutant HS1A15, show an absolute requirement for glutamate. Thus, selection for *cit*⁺ transformants was done by plating on minimal glucose medium. Strain HS1A15 is able to grow, albeit slowly, without glutamate. *cit*⁺ transformants of this strain can be scored on minimal glucose plates as large, pigmented colonies, whereas the *cit*⁻ recipient colonies are small and unpigmented. Enzymatic analysis showed that the presumptive *cit*⁺ transformants had wild-type levels of isocitrate dehydrogenase. The ratio *cit*⁺ to *trp*⁺ was calculated for each donor, and the recombination index was defined as the ratio *cit*⁺/*trp*⁺ (*cit* donor) divided by the ratio *cit*⁺/*trp*⁺ (wild-type donor).

RESULTS

Repression of aconitase by glutamate in the wild-type strain. The previous observation that aconitase is repressed by glutamate was confirmed in the experiment shown in Fig. 1. In unsupplemented NSMP medium, the level of aconitase increased during growth and reached a sharp maximum in late exponential phase. The addition of 5 mM glutamate prevented this increase almost completely.

As shown in earlier studies (13, 14) and in Fig. 1A, the wild-type strain has a biphasic growth curve in NSMP medium. Unsupplemented NSMP medium contains about 0.1 mM glutamate (30), and the growth lag in mid-exponential phase reflects adaptation to glutamate (13; unpublished data). In unsupplemented NSMP medium, adaptation to glutamate is followed by a period of repression of aconitase (Fig. 1B).

In minimal glucose medium, the level of aconitase was reduced to about 15% of the maximum level observed in NSMP medium (Fig. 2B). This depends on catabolite repression, which will be discussed in a later paragraph. No further repression of aconitase was seen when minimal glucose medium was supplemented with 5 mM glutamate.

Induction of aconitase by citrate and aspartate in the wild-type strain growing in NSMP medium. The addition of 5 mM citrate to NSMP medium was found to have a marked influence on aconitase in the wild-type strain (Fig. 1B). In a culture growing with citrate, the level of aconitase started to increase at least two generations earlier than in a control culture lacking citrate, and less repression of aconitase followed adaptation to glutamate. In the presence of citrate, aconitase activity reached a higher level than in the control culture.

A number of control experiments (data now shown) indicated that the effect of citrate on the levels of aconitase depends on induction of aconitase synthesis. The presence of 5 mM citrate did not affect the growth rate or the rate of total protein synthesis. The levels of two other citric acid cycle enzymes, isocitrate dehydrogenase and fumarase, were unaffected by the addition of citrate. Citric acid cycle intermediates other than citrate had no effect on the level of aconitase when tested at a concentration of 5 mM. The only metabolite tested, except citrate, that increased the level of aconitase was aspartate (Table 4). This amino acid can be converted to citrate by the action of glutamate-oxalacetate transaminase and citrate synthase.

Induction of aconitase by citrate and precursors of citrate in the wild-type strain growing in minimal glucose medium. The experiments reported in the preceding paragraph were repeated with wild-type cells growing in minimal glucose medium. In the presence of 5 mM citrate, the level of aconitase increased about fourfold during the exponential phase, whereas no increase in the level of aconitase was seen in the control culture lacking citrate (Fig. 2B).

The effects of other citric acid cycle intermediates and related metabolites on aconitase synthesis in minimal glucose medium was investi-

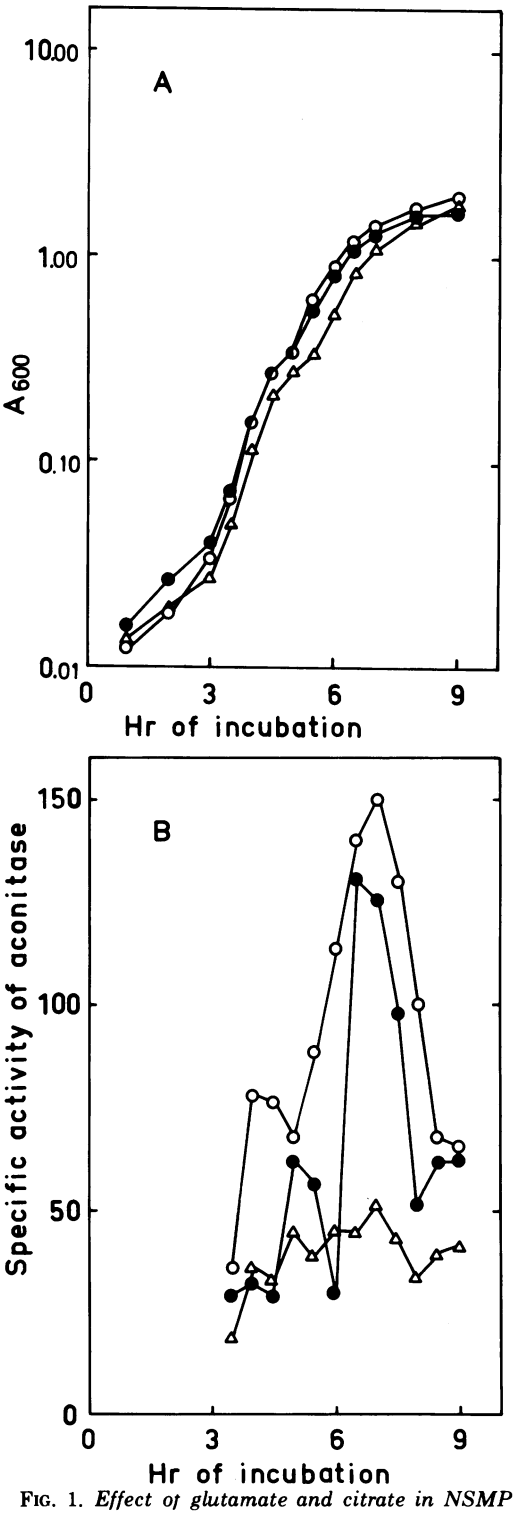

FIG. 1. *Effect of glutamate and citrate in NSMP medium on (A) growth and (B) specific activity of aconitase in B. subtilis wild type. Symbols: ●, no supplement; △, 5 mM glutamate; ○, 5 mM citrate.*

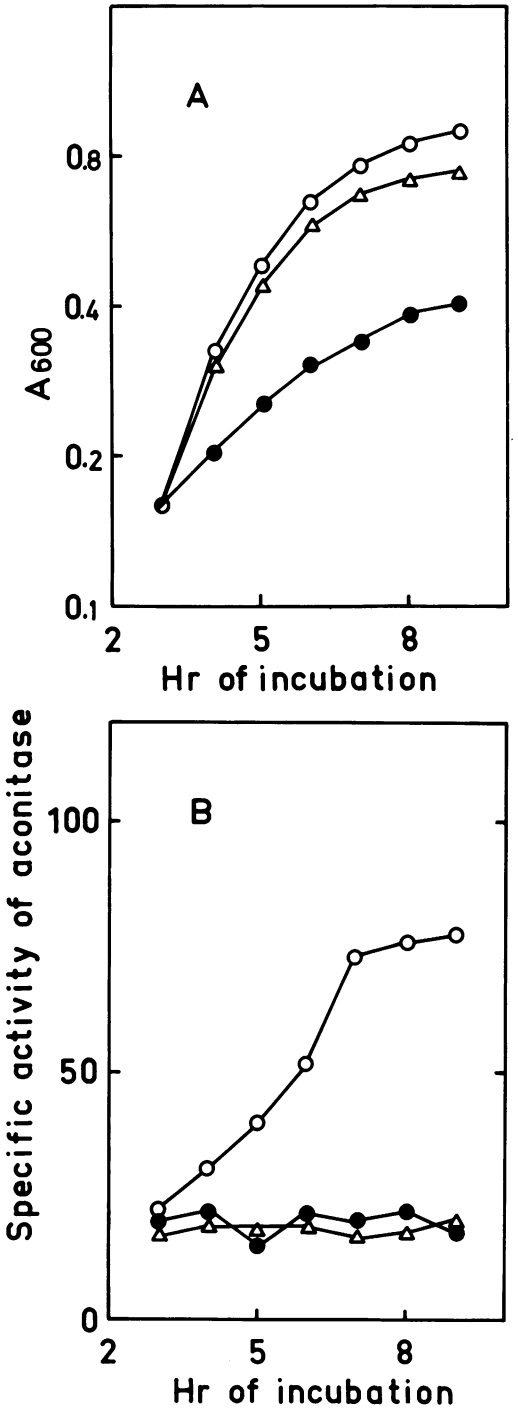

FIG. 2. *Effect of glutamate and citrate in minimal glucose medium on (A) growth and (B) specific activity of aconitase in B. subtilis wild type. Symbols: ●, no supplement; △, 5 mM glutamate; ○, 5 mM citrate.*

gated. The levels of isocitrate dehydrogenase and fumarase were also measured. The results are summarized in Table 2. All of the metabolites tested, except glutamate, stimulated aconitase synthesis. The activity of aconitase was highest in the cultures containing citrate, fumarate, or aspartate. The strong effect of fumarate on aconitase is most likely due to the direct conversion of fumarate to aspartate by aspartase (12). This also explains why fumarate has a stronger effect on aconitase synthesis than malate.

The growth rate was increased two- to threefold in the cultures supplemented with citrate or glutamate (Fig. 2A). The other supplements tested did not affect the growth rate. There is thus no strict correlation between the stimulation of growth and the stimulation of aconitase synthesis.

The addition of aspartate caused a slight increase in the level of isocitrate dehydrogenase. The reason for this finding is not known. In agreement with earlier observations (9, 17), the synthesis of isocitrate dehydrogenase was seen to be repressed by glutamate. The synthesis of fumarase was not significantly affected by any supplements.

Endogenous induction of aconitase in isocitrate dehydrogenase mutants. If citrate acts as an inducer of aconitase synthesis, it should be possible to demonstrate this in citrate-accumulating mutants. It has been shown that mutants defective in isocitrate dehydrogenase accumulate citrate and isocitrate (5, 10, 28); these compounds could not be separated in the thin-layer chromatography system used. Citrate is most likely the main intermediate accu-

TABLE 2. *Effect of different supplements to minimal glucose medium on the specific activities of aconitase, isocitrate dehydrogenase, and fumarase in B. subtilis wild type*

Supplement (5 mM)	A_{600}[a]	Sp act		
		Aconitase	Isocitrate dehydrogenase	Fumarase
None	0.45	23	187	367
Pyruvate	0.45	38	173	362
Citrate	0.90	75	199	347
Glutamate	0.80	23	103	357
Succinate	0.45	37	168	337
Fumarate	0.45	64	173	394
Malate	0.50	47	178	388
Aspartate	0.50	60	225	385

[a] Bacteria were harvested at the end of the exponential growth phase.

mulated in these mutants, because the aconitase equilibrium is in the direction of citrate production (21).

The level of aconitase was found to be about 10-fold higher in CitC3 (Idh^-) than in the wild-type strain and was further increased by the addition of 5 mM citrate (Fig. 3B) or aspartate (data not shown). Similar results were obtained with four other Idh^- mutants, CitC1, CitC6, HS1A15, and HS2A2. This provides clear evidence for induction of aconitase by citrate. Citrate-accumulating mutants are also endogenously induced for citrate uptake (31).

Unexpectedly, aconitase was not repressed by 5 mM glutamate in any of the Idh^- mutants. The strong endogenous induction apparently overcomes repression by glutamate. In fact, the level of aconitase was increased by the addition of glutamate (Fig. 3B), which must depend on metabolism of glutamate to citrate. Isocitrate dehydrogenase mutants are not defective in glutamate uptake. I have found that the initial rate of [^{14}C]glutamate uptake by whole cells is about twofold higher in Idh^- mutants than in the wild type (unpublished data). Glutamate uptake was measured by the method of Fortnagel and Freese (10).

Effect of citrate on the in vitro activity of aconitase. The possibility that intracellular citrate acts by stabilizing or activating aconitase during the preparation of extracts was considered. When wild-type extracts were prepared in buffer containing chloramphenicol plus 5 mM citrate, a 15 to 20% increase in the specific activity of aconitase was noted. This is not sufficient to account for the great difference in the level of aconitase between cells grown in the presence and in the absence of citrate. It thus seems reasonable to assume that the effect of citrate is primarily due to increased synthesis of enzyme protein and, to a lesser extent, is caused by activation or stabilization of the enzyme. Activation by citrate was obtained with extracts from all mutants studied, including the citrate-accumulating isocitrate dehydrogenase mutants.

Dependence of aconitase synthesis on the concentration of citrate and glutamate. Figure 4 shows the specific activity of aconitase as a function of the concentration of citrate and glutamate. Exponentially growing cells were incubated with different concentrations of citrate in the absence of glutamate or in the presence of 2.5 mM glutamate. Different concentrations of glutamate were also tested in the absence of citrate or in the presence of 2.5 mM citrate. The time of incubation was confined to one generation to prevent extensive metabolism of citrate and glutamate. Neither the growth rate nor the rate of total protein synthesis was affected by the supplements.

The level of aconitase was increased 120% by 5 mM citrate. The same concentration of glutamate reduced the level of aconitase to 30% of the control value. The effects of citrate and glutamate at concentrations higher than 5 mM varied between different experiments, and data are now shown.

One interesting observation is that at equimolar concentrations (2.5 mM) of citrate and glutamate, aconitase synthesis was completely unaffected. Induction predominated in the presence of 5 mM citrate and 2.5 mM glutamate, and repression predominated at 5 mM glutamate and 2.5 mM citrate. It should be pointed out that the rates of uptake and metabolism of citrate and glutamate may be different. The relative concentrations of the two effectors are therefore not necessarily the same inside the cell as in the growth medium. It should also be taken into account that unsupplemented NSMP medium probably contains both citrate and glutamate. The concentration of glutamate may be as high as 0.1 mM (30) although the concentration of citrate is not known.

Catabolite repression of aconitase. Earlier studies (6, 15–17) have indicated that aconitase synthesis in *B. subtilis* is subject to catabolite repression. This has been shown by measurements of aconitase levels in cells grown in minimal medium with different carbon sources.

Repression is also seen in cells grown in NSMP medium plus 0.5% glucose (Fig. 5B). It is worth noting that aconitase was repressed to the same level in the wild-type and in the citrate-accumulating mutant CitC3 (Idh^-) (Fig. 6B). The addition of citrate partly relieved glucose repression in the mutant but not in the wild type. This experiment is complicated by the finding that citrate uptake is sensitive to catabolite repression (31). It is clear, however, that some citrate is actually taken up in the presence of glucose at least in CitC3, because citrate has an inductive effect in this strain.

Effect of citrate on aconitase synthesis in leaky aconitase mutants. Mutants HS1A14 and HS2A1 are defective in both citrate synthase and aconitase (5). This phenotype presumably results from a single mutation, because revertants of these strains have regained normal levels of both enzymes. Low levels of aconitase could be detected in HS1A14 and HS2A1 as well as in the two mutants defective in aconitase only, HS3A20 and CitB25. The data given in Table 3 show that addition of citrate increased the synthesis of aconitase in

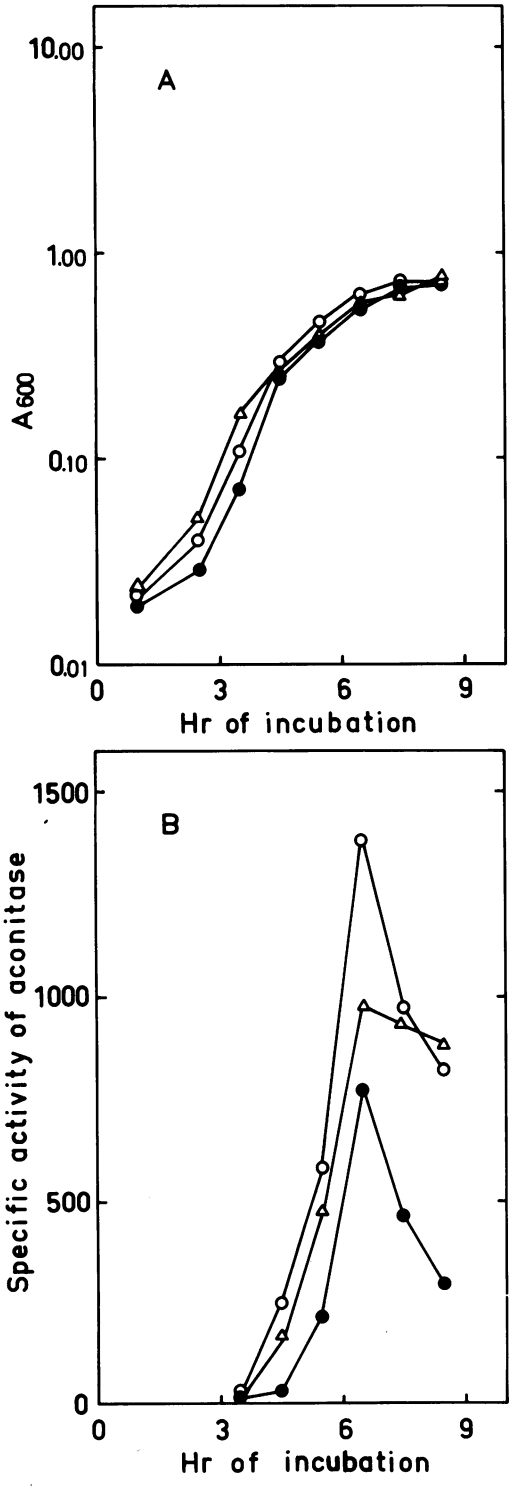

FIG. 3. *Effect of glutamate and citrate in NSMP medium on (A) growth and (B) specific activity of aconitase in B. subtilis CitC3 (Idh⁻). Symbols: ●, no supplement; △, 5 mM glutamate; ○, 5 mM citrate.*

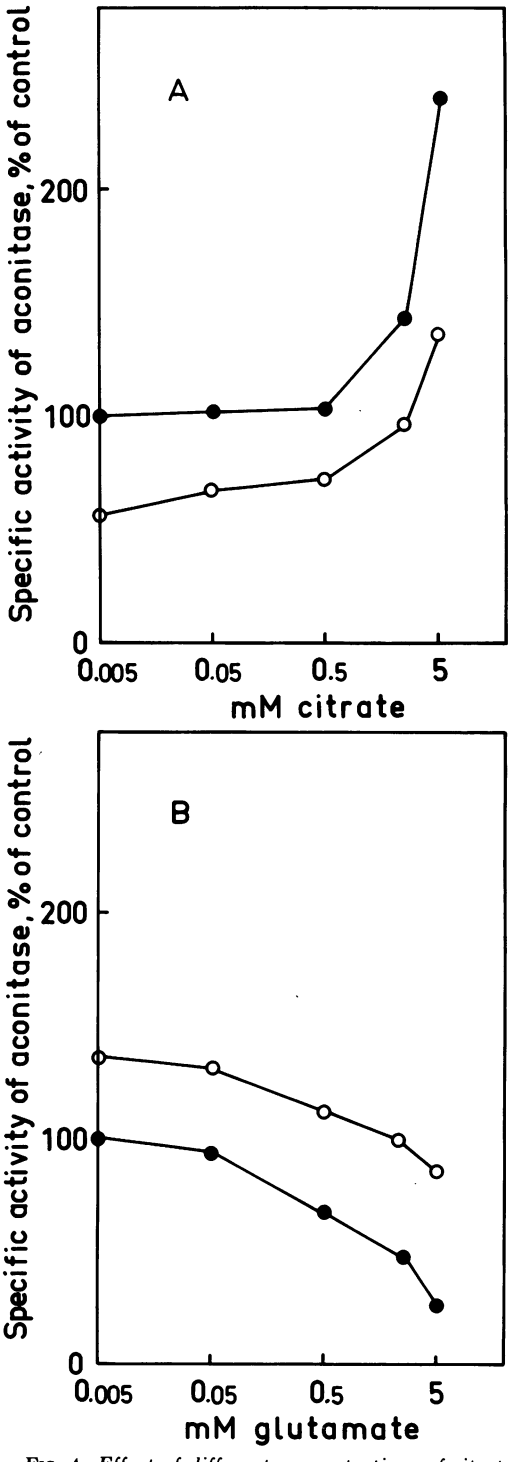

FIG. 4. *Effect of different concentrations of citrate and glutamate in NSMP medium on the specific activity of aconitase in B. subtilis wild type. Supplements were added at A_{600} = 0.30 and cells were harvested at A_{600} = 0.60. No supplements were added to the control culture. A, Different concentrations of citrate. Symbols: ●, no glutamate; ○, 2.5 mM glutamate. B, Different concentrations of glutamate. Symbols: ●, no citrate; ○, 2.5 mM citrate.*

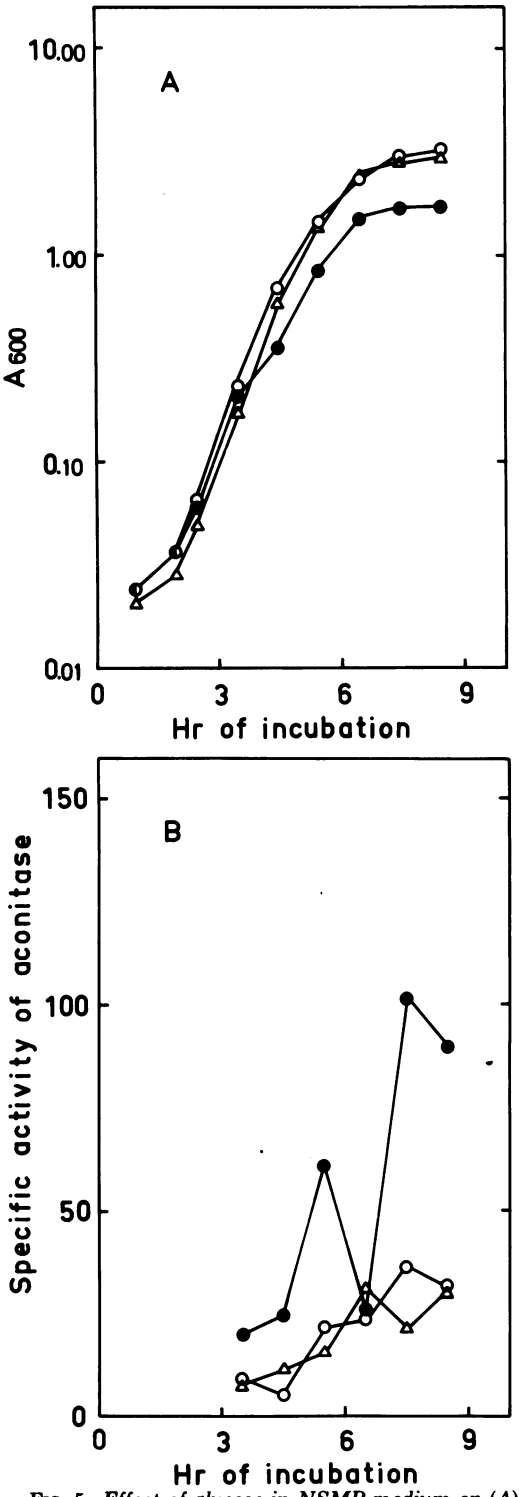

FIG. 5. *Effect of glucose in NSMP medium on (A) growth and (B) specific activity of aconitase in B. subtilis wild type. Symbols: ●, no supplement; ○, 0.5% glucose; △, 0.5% glucose plus 5 mM citrate.*

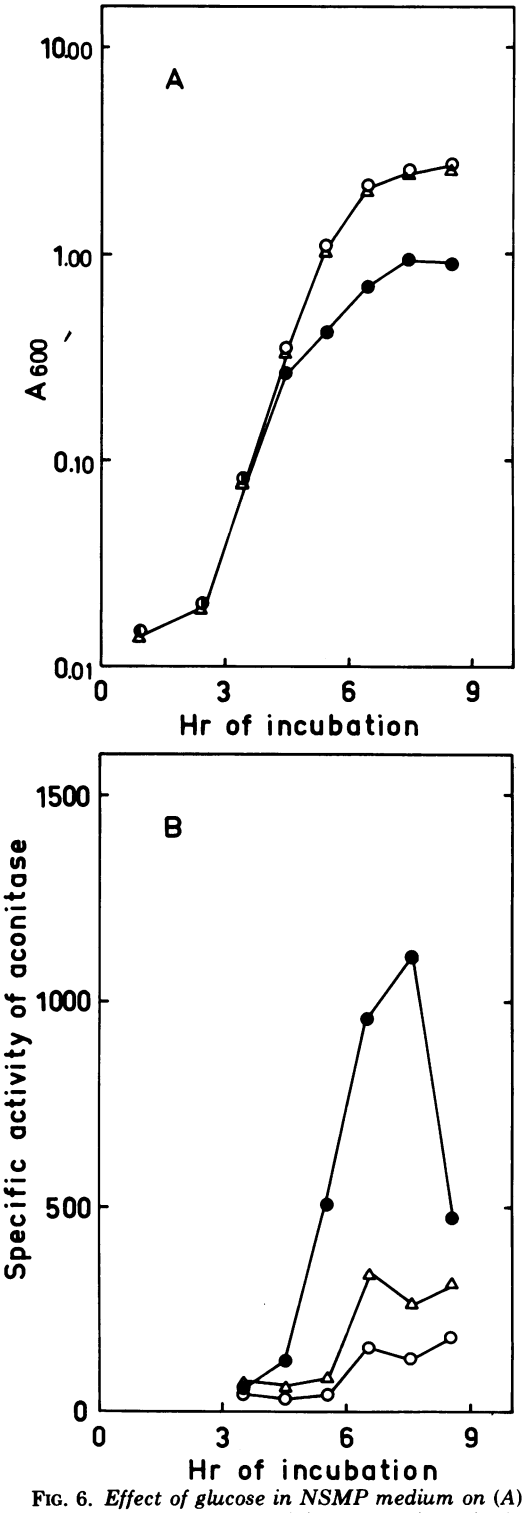

FIG. 6. *Effect of glucose in NSMP medium on (A) growth and (B) specific activity of aconitase in B. subtilis CitC3 (Idh⁻). Symbols: ●, no supplement; ○, 0.5% glucose; △, 0.5% glucose plus 5 mM citrate.*

TABLE 3. *Effect of citrate on the specific activity of aconitase in citric acid cycle mutants defective in citrate synthase and aconitase and in aconitase*

Strain[a]	Phenotype[b]	Sp act of aconitase	
		No supplement	Citrate (5 mM)
HS1A14	Cts⁻ Aco⁻	12	11
HS2A1	Cts⁻ Aco⁻	4	3
HS3A20	Aco⁻	1	4
CitB25	Aco⁻	1	3

[a] Bacteria were grown in NSMP medium and were harvested at A_{600} = 0.50.

[b] For phenotype symbols, see Table 1.

HS3A20 and CitB25 but not in HS1A14 or HS2A1. This finding indicates that mutants HS1A14 and HS2A1 are defective in the regulation of aconitase synthesis. Studies on repression were not possible with these strains because of the low basal activities.

Induction of aconitase in mutants defective in α-ketoglutarate dehydrogenase, succinate dehydrogenase, or fumarase. Citric acid cycle mutants accumulating α-ketoglutarate, succinate, fumarate, or malate show reduced levels of aconitase (5, 9, 25, 28). An attempt was made to induce aconitase in such mutants by growing them in the presence of citrate or aspartate (Table 4). The growth rates of the mutants were not altered by the supplements. Aconitase could be induced in all four mutants studied; however, the induced levels in the mutants were generally lower than the induced level in the wild type. This finding supports the idea that the internal concentration of α-ketoglutarate or glutamate is elevated in these mutants (25). Aspartate was found to be a strikingly effective inducer in α-ketoglutarate dehydrogenase mutants. This observation cannot be explained at present, but it is possible that aspartate uptake is elevated in α-ketoglutarate dehydrogenase mutants compared with the wild type.

Effect of different compounds on the activity of aconitase in vitro. A number of compounds were screened for effects on aconitase activity in vitro. An extract of the wild type grown in NSMP medium was incubated with the compound to be tested in 50 mM potassium phosphate buffer, pH 7.4, for 3 min at room temperature. Aconitase activity was then measured after the addition of isocitrate. The following compounds were tested at final concentrations of 0.1 mM and 1.0 mM: α-ketoglutarate, succinate, fumarate, malate, oxalacetate, pyruvate, acetate, glyoxylate, glutamate, and aspartate. None of the compounds tested had any effect on the activity of aconitase. The effect of malate was investigated by using an extract from a fumarase-negative mutant, because otherwise the fumarate synthesized from malate by fumarase causes an increase in the absorbance at 240 nm, which interferes with the aconitase assay.

Complementation tests. Strains HS1A14 and HS2A1 (Cts⁻ Aco⁻) and HS3A20 and CitB25 (Aco⁻) were tested for in vitro complementation of aconitase in all possible pairwise combinations. Equal amounts of extracts from the two mutants to be tested were mixed, and aconitase activity was assayed. Activity was not observed in any of the combinations.

The activity of aconitase in a wild-type extract was not affected by the addition of extract from any of the mutants HS1A14, HS2A1, HS3A20, or CitB25, which suggests that these mutants do not produce an inhibitor of aconitase activity.

Genetic analysis. The *citC* locus (isocitrate dehydrogenase) has been shown to be located between *argA* and *citF* on the *B. subtilis* chromosome (28). The *citB* locus (aconitase) was placed between *gap* and *thyA* on the genetic map constructed by Young and Wilson (32), but experimental data supporting this location have not been published.

The recombination index method (see Materials and Methods) was used to uncover linkage between previously located *cit* markers and markers carried by the HS strains, which have not been subject to genetic analysis before. I found that strain HS1A14 (Cts⁻ Aco⁻) and strain CitB25 (Aco⁻) carry mutations which are linked to each other, whereas the mutations carried by strains HS1A15 and HS2A2 (Idh⁻) are linked to the *citC* locus (Table 5).

TABLE 4. *Induction of aconitase by citrate and aspartate in citric acid cycle mutants defective in α-ketoglutarate dehydrogenase, succinate dehydrogenase, or fumarase*

Strain[a]	Phenotype[b]	Sp act of aconitase		
		No supplement	Citrate (5 mM)	Aspartate (5 mM)
Wild type	Cit⁺	56	112	87
CitD29	Kdh⁻	34	47	108
CitK14	Kdh⁻	33	59	152
CitF8	Sdh⁻	13	81	30
CitG4	Fum⁻	16	60	36

[a] Bacteria were grown in NSMP medium and were harvested at A_{600} = 0.30.

[b] For phenotype symbols, see Table 1.

TABLE 5. *Genetic analysis of citric acid cycle mutants by the recombination index method*[a]

Donor strain	Recipient strain	Recombination (%)[b]
HS1A14	CitB25	9.2
HS1A14	CitC6	100
CitB25	HS1A14	11.0
HS1A15	CitB25	100
HS1A15	CitC6	5.2
CitC6	HS1A15	6.3
HS2A2	CitB25	100
HS2A2	CitC6	2.4
CitC6	HS2A2	1.4
HS1A15	HS2A2	9.0

[a] Recombination index analysis was carried out as described in Materials and Methods.

[b] Percent recombination was calculated as the recombination index $\times 100$.

DISCUSSION

The results presented in this paper show that the synthesis of aconitase in *B. subtilis* is induced by aspartate and citrate and repressed by glutamate.

Aspartate can be converted to citrate by the combined action of glutamate-oxalacetate transaminase and citrate synthase. Conversion of citrate to aspartate does not occur, since citrate lyase is missing in *B. subtilis* (10, 31). Citrate is therefore most likely the true inducer. This conclusion is supported by the finding (5) that a citrate synthase mutant which accumulates aspartate but has very small amounts of citrate (or isocitrate) has about 10% or less of the wild-type level of aconitase. However, the rather unlikely possibility that *cis*-aconitate or isocitrate is the true inducer cannot be excluded at present.

Inducibility of aconitase in *B. subtilis* has been suggested by earlier observations. Hanson et al. (15) found that repression of aconitase by glutamate could be partially alleviated by aspartate. In a study on catabolite repression of aconitase, Cox and Hanson (6) remarked upon the similarity of aconitase to other catabolite-sensitive enzymes known to be inducible. These authors could not obtain evidence for induction of aconitase, but since they used basal growth media containing 0.1% (3.4 mM) citrate, the cells were actually grown under inducing conditions in all experiments.

The high levels of aconitase in mutants defective in isocitrate dehydrogenase were previously thought to be due to derepression of aconitase in the absence of glutamate biosynthesis (5, 28). Fortnagel (9) reported that aconitase production in an isocitrate dehydrogenase mutant could not be repressed to wild-type levels by the addition of glutamate. The present study shows that the accumulation of citrate in such mutants results in endogenous induction of aconitase overriding repression by glutamate.

Glutamate will conceivably affect aconitase synthesis indirectly by limiting the endogenous formation of citrate, as citrate synthase is sensitive to feedback repression by glutamate (or a metabolite derived from glutamate) (7). I have found, however, that glutamate also prevents induction of aconitase by exogenous citrate, i.e., that glutamate interferes with aconitase synthesis under conditions where the activity of citrate synthase is irrelevant. I conclude, in agreement with other investigators, that aconitase is indeed a repressible enzyme. Evidence has been obtained that α-ketoglutarate is the true corepressor for aconitase (J. A. Hoch, personal communication). The effect of α-ketoglutarate on aconitase synthesis has not been studied here because α-ketoglutarate is not taken up at sufficient rate by the wild-type strain used in this laboratory.

The wild-type level of aconitase changes drastically during logarithmic growth in a complex medium, presumably reflecting rapid fluctuations in the citrate to glutamate ratio. To obtain a full picture of the regulation of aconitase, it is necessary to follow enzyme synthesis continuously during growth. In this study, single-point data (Tables 3 and 4) are given only for mutants in which aconitase synthesis does not change significantly during growth (this was determined in preliminary experiments).

The synthesis of aconitase is sensitive to catabolite repression. It has been shown here that aconitase is repressed by glucose in an otherwise endogenously induced isocitrate dehydrogenase mutant. Citrate addition partially overcomes glucose repression in this mutant. The most reasonable interpretation is that catabolite repression interferes with induction of aconitase by citrate. This mechanism would be analogous to classical catabolite repression of enzymes of degradative pathways such as β-galactosidase (23). Again, indirect effects, i.e., repression of enzymes responsible for citrate synthesis and glutamate metabolism, can also be expected. Cox and Hanson (6) have suggested that the effector of catabolite repression of aconitase in *B. subtilis* is metabolically related to adenosine 5'-triphosphate (ATP).

The fine control of aconitase has been little studied previously. Fortnagel and Freese (11) have reported that aconitase is stabilized by ferrous ions and noncompetitively inhibited by chelators. The present study shows that aconi-

tase is also stabilized or activated by citrate. I have not found any evidence for feedback inhibition of aconitase by citric acid cycle intermediates. Crude extracts were used for these studies and it cannot be excluded that purified enzyme preparations would respond differently.

The level of aconitase under any particular growth conditions will be determined by the intracellular concentrations of citrate, glutamate, and effector(s) of catabolite repression. Aconitase participates in energy production as well as in glutamate biosynthesis. This dual role of aconitase provides a rationale for its mode of regulation. Induction of aconitase by citrate ensures that ATP production via the citric acid cycle continues even in the presence of adequate amounts of glutamate, provided a suitable substrate (citrate or any intermediate capable of being converted to citrate) is available. This may be particularly important during sporulation when ATP production via the citric acid cycle must proceed concomitantly with glutamate accumulation. Both these processes seem to be indispensable to sporulation. Mutants blocked in the synthesis of ATP via the citric acid cycle are unable to sporulate (10, 19). Glutamate is accumulated late in sporulation in at least three *Bacillus* species, including *B. subtilis*, and makes up 1% of the dry weight and 75% of the total free amino acids of the mature spore (24). It has been suggested that glutamate functions as a chelator of divalent cations in the spore (24) or as a source of γ-aminobutyrate during germination (8).

During growth in media containing glucose, the energy requirement can be met with ATP production by glycolysis, and the synthesis of the citric acid cycle enzymes is repressed. Catabolite repression is only partial (16) so that the biosynthetic role of the cycle is fulfilled also in the presence of glucose.

Flechtner and Hanson (7) have proposed a coordinate regulation of citrate synthase and aconitase in *B. subtilis* because of the similar kinetics of glutamate repression and derepression of the two enzymes. This was supported by the isolation (5) of two mutants, HS1A14 and HS2A1, with reduced levels of both enzymes. The finding that aconitase is not inducible in these mutants points to a regulatory defect. I have experienced difficulties in assaying citrate synthase in crude extracts of *B. subtilis* and citrate synthase activities have not been determined in this study. Therefore, I do not know if strains HS1A14 and HS2A1 are defective in the regulation of citrate synthase as well. The hypothesis that citrate synthase and aconitase are not only coordinately repressed, but also coordinately induced, is attractive but can be neither supported nor rejected on the basis of existing data.

The two leaky aconitase mutants, HS3A20 and CitB25, show normal inducibility of aconitase although the specific activities are severely reduced. These strains may be mutated in the structural gene for aconitase. It is interesting that the mutations carried by HS1A14 (Cts^- Aco^-) and CitB25 (Aco^-) are linked. However, all attempts to design a model for the regulation of citrate synthase and aconitase at the genetic level would be mere speculations at the moment.

I am not aware of any previously characterized regulatory system with the same properties as the one described here. I hope that the further exploration of the regulation of aconitase synthesis in *B. subtilis* will contribute to our understanding of the control of amphibolic pathways in bacteria.

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