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Induction of Citric Acid Cycle Enzymes During Initiation of Sporulation by Guanine Nucleotide Deprivation

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In *Bacillus subtilis*, conditions causing partial deprivation of guanine nucleotides initiated sporulation and caused the synthesis of citrate synthase, aconitase, and alpha-ketoglutarate dehydrogenase. Alpha-ketoglutarate dehydrogenase could also be induced by acetate, and the specific activity of this enzyme was elevated in mutants that had high intracellular acetyl coenzyme A concentrations because they lacked citrate synthase activity. After deprivation of guanine nucleotides, the intracellular concentration of acetyl coenzyme A also increased, which explained the induction of alpha-ketoglutarate dehydrogenase. Furthermore, the decreases in alpha-ketoglutarate and L-malate concentrations observed during this deprivation accounted for the observed increases in citrate synthase activity (which was repressed by alpha-ketoglutarate and malate) and aconitase activity (which was repressed by alpha-ketoglutarate).

When growth is curtailed severely because rapidly metabolizable nutrients are exhausted, wild-type strains of *Bacillus subtilis* develop heat-resistant endospores. This sporulation process is accompanied by numerous enzymatic changes. Because nutrient limitation reduces the synthesis of most metabolites, it is difficult to determine which of these enzymatic changes are associated with the sporulation process and which reflect unrelated metabolic alterations.

Previous work from our laboratory has shown that cells growing exponentially in rich media (containing excess glucose) can be induced to sporulate by the specific and partial inhibition of guanine nucleotide synthesis. This inhibition, which also reduces the increase in cell mass, can be obtained by adding inhibitors of purine synthesis (13) (e.g., decoyinine, an inhibitor of GMP synthetase [EC 6.3.4.1] [18]) or by starving purine (especially guanine) auxotrophs (6, 7) for purines. When such inducing conditions are used in the presence of rapidly metabolizable carbon sources, only those enzymatic changes should occur which are associated with (although not necessarily required for) the sporulation process. Following this rationale, we have shown previously that the effects of glucose on sporulation and on the synthesis of several inducible enzymes (catabolite repression) do not result from the same mechanism; whereas sporulation can be induced in the presence of glucose, the synthesis of three inducible enzymes remains repressed (17).

The specific activities of several enzymes of the citric acid cycle increase at the beginning of spore development in carbon-limited media (12). We report here that the specific activities of citrate synthase, aconitase, and alpha-ketoglutarate dehydrogenase also increased considerably in a glucose-containing medium shortly after decoyinine addition to the standard strain or after guanosine withdrawal from a guanine auxotroph under conditions which initiated sporulation. We compare the increases in enzyme specific activities and metabolite concentrations during GMP deprivation with the corresponding changes in various mutants and after addition of acetate. Our results show that the sporulation-related increases in the specific activities of these enzymes can be explained by changes in the concentrations of metabolites, such as acetyl coenzyme A (acetyl-CoA), alpha-ketoglutarate, and malate.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *B. subtilis* used are listed in Table 1. They were grown in synthetic medium containing 100 mM glucose and a mixture of most amino acids (16) from an inoculum (initial optical density at 600 nm [OD_{600}], 0.05) from a culture grown overnight on a plate containing tryptose blood agar base (Difco Laboratories, Detroit, Mich.). Growth was measured by determining increases in the OD_{600}.

Chemicals. Decoyinine was a gift from G. B. Whitefield, The Upjohn Co., Kalamazoo, Mich. Carrier-free $Na_2{}^{35}SO_4$ was purchased from New England Nuclear Corp., Boston, Mass.

Preparation of extracts for enzyme assays. Bacteria (20 ml) were harvested by centrifugation, washed twice with ice-cold 0.05 M potassium phos-

TABLE 1. *B. subtilis strains used in this work*

Strain	Source	Relevant enzyme defect(s)	Genotype
60005	This laboratory	None	Prototroph
60015	This laboratory	None	*metC7 trpC2*
60871	This laboratory	Aconitase and citrate synthase	*citB metC7 trpC2*
61676	This laboratory	IMP dehydrogenase	*guaA metC7 purH trpC2*
61423	Hanson	Fumarase	*citG trpC*
61461	Hoch	Malate dehydrogenase	*citH trpC*
61861	Hanson	Citrate synthase	*citA leu met*

phate buffer (pH 8.0)–0.15 M NaCl, and then suspended in 0.9 ml of the same solution. After 0.1 ml of lysozyme (1 mg/ml) was added, the suspension was incubated for 20 min at 37°C. A cell extract was obtained by centrifuging the suspension for 20 min at 48,000 × *g*. Enzymes were assayed on the same day.

Proteins were determined by the method of Kalb and Bernlohr (15) by measuring the absorbances at 230 and 260 nm.

Enzyme assays. Enzymes were assayed at room temperature with a Gilford spectrophotometer. Specific activities were calculated as nanomoles of substrate converted per minute per milligram of protein.

The reaction mixture used for the determination of citrate synthase (EC 4.1.3.7) activity contained 0.1 M Tris-chloride (pH 8.0), 10 mM potassium malate, 6 mM NAD, 100 U of malate dehydrogenase per ml, 0.2 mg of acetyl-CoA per ml, and cell-free extract. After acetyl-CoA was added, the increase in absorbance at 340 nm was recorded for several minutes, and the linear part of the slope was used to calculate the specific activity.

Aconitase (EC 4.2.1.3) was assayed as described by Hanson and Cox (10), except that 100 mM sodium isocitrate was used.

Alpha-ketoglutarate dehydrogenase (EC 1.2.4.2) was assayed by the method of Ohné (20). Pyruvate dehydrogenase (EC 1.2.4.1) activity was measured by the method used for the alpha-ketoglutarate dehydrogenase assay, except that pyruvate was used as the substrate.

Determination of acetyl-CoA concentrations. Cells were grown in synthetic glucose medium in which $(NH_4)_2SO_4$ was replaced by NH_4Cl; also, this medium contained only 0.05 μM K_2SO_4. Carrier-free $Na_2{}^{35}SO_4$ (200 μCi/ml) was added when the OD_{600} was 0.1. At the times indicated on the figures, the cells from 0.75-ml portions of cultures were collected on membrane filters (pore size, 0.45 μm; diameter, 2.5 cm; Millipore Corp., Bedford, Mass.) under suction, and each filter was laid upside down on 100 μl of ice-cold 0.75 M formic acid located in the cap of a 50-ml centrifuge tube (Corning). The whole sampling procedure took about 8 s. After 60 to 90 min in a refrigerator, each capped tube containing a filter was centrifuged for 5 min at approximately 8,000 × *g*, and the supernatant was transferred to another tube. A 5-μl portion of this supernatant was applied to one corner of a thin-layer cellulose plate (20 by 20 cm; thickness, 0.1 mm; MN-Polygram Cel 400 UV; Brinkmann Instruments Inc., Westbury, N.Y.). Standard CoA compounds (10 μg each of CoA, malonyl-CoA, acetyl-CoA, and *n*-propionyl-CoA) had been applied to the same point onto which the sample was spotted. The plate was developed in a solution containing isobutyric acid, concentrated NH_4OH, and water (66:1:33) in the first dimension and, after drying, in a solution containing *n*-butanol, glacial acetic acid, and water (4:1:1) in the second dimension. After complete drying and marking with radioactive ink spots, the plates were placed against X-Omat R X-ray film (Kodak Co.) and incubated for 40 h. Radioactive spots were cut out and counted in scintillation vials containing 10 ml of Aquasol. The results were expressed in femtomoles per AM_{600} unit; 1 AM_{600} unit was the amount of cells which, if contained in 1 ml, gave an OD_{600} of 1.0.

Determination of alpha-ketoglutarate, malate, and pyruvate concentrations. Cells were grown in synthetic medium to an OD_{600} of 0.5. The culture was divided into two parts. One part received 1.44 mM decoyinine, and the other did not. Samples containing 50 AM_{600} units were taken before the division and at different times thereafter. Each sample was immediately filtered through a Millipore filter (pore size, 0.45 μm; diameter, 9 cm), washed twice with 10 ml of prewarmed growth medium, and then laid upside down on 1 ml of ice-cold 0.75 M formic acid in a plastic petri dish. The total time from the start of sample collection to the immersion of the filter in acid was less than 30 s. After 60 min in a refrigerator, the formic acid was collected with a Pasteur pipette, and the bacteria-containing side of the filter was washed with 1 ml of water.The total extract collected was centrifuged, and the supernatant was freeze-dried overnight. The freeze-dried extract was then dissolved in 0.1 M Tris-chloride (pH 8.0) and used for the determinations described below.

The reaction mixture for the determination of alpha-ketoglutarate concentration contained (in 0.5 ml) 0.3 mM NADH, 0.1 M ammonium sulfate, 0.2 M Tris-chloride (pH 8.0), 0.025 U of glutamate dehydrogenase, and cell extract. The reaction mixture for the determination of malate concentration contained 0.43 M glycine, 0.34 M hydrazine (pH 9.0), 2.75 mM NAD, 50 U of malate dehydrogenase, and extract (14). The reaction mixture for the determination of pyruvate concentration contained 0.3 mM NADH, 0.2 M Tris-chloride (pH 8.0), 0.35 U of lactate dehydrogenase, and extract. The concentrations of these metabolites were calculated from the differences between the absorbance values at 340 nm before enzymes were added and the new stationary absorbance values reached after the addition of the enzymes, using a millimolar extinction coefficient for NADH of 6.22.

RESULTS

Induction of citrate synthase, aconitase, and alpha-ketoglutarate dehydrogenase by limitation of guanine nucleotides. To determine whether the specific activities of certain citric acid cycle enzymes increased under conditions of partial deprivation of guanine nucleotide synthesis, we either added 1.44 mM decoy-

inine to a culture of the standard strain (strain 60015) or removed guanosine from a culture of a guanine auxotroph (strain 61676 [*purH guaA*]). We grew the cells in a synthetic medium containing excess ammonium ions, glucose, and phosphate; the cells could sporulate in this medium only when the synthesis of guanine nucleotides was limited. After decoyinine was added, 20 to 30% of the cells produced heat-resistant spores, and after guanosine was removed, 10 to 20% of the cells produced spores (measured 10 h after the limitation was initiated). If a small amount of guanosine (50 μM) had been added to the culture of the guanine auxotroph, better sporulation would have been obtained (6), but the deprivation of guanine nucleotides would have started later and at a less-well-defined time.

For the decoyinine experiment, a culture of standard strain 60015 growing exponentially in synthetic medium was divided into two parts when the OD_{600} was 0.5. One part received 1.44 mM decoyinine, and the other did not. Just before the division of the culture and at different times thereafter, 20-ml samples were collected, the cells were washed, and extracts were prepared as described above. Decoyinine addition caused increases in the specific activities of citrate synthase, aconitase, and alpha-ketoglutarate dehydrogenase by factors of 10 or more (Fig. 1) and a threefold increase in the isocitrate dehydrogenase specific activity (data not shown). In the control culture (without decoyinine), the specific activities of citrate synthase and aconitase did not change significantly, whereas the specific activity of alpha-ketoglutarate dehydrogenase increased by less than a factor of four. If rifampin (10 μg/ml) was added simultaneously with decoyinine, the specific activities of citrate synthase, aconitase, and alpha-ketoglutarate dehydrogenase did not increase.

For guanosine deprivation, the guanine auxotroph strain 61676 (*purH guaA*) was grown in synthetic medium containing 1 mM guanosine and 740 μM adenosine (this satisfied the general purine requirement of strain 61676). At an OD_{600} of 0.5, the bacteria were collected by Millipore filtration and then suspended (to give the same OD_{600}) in prewarmed synthetic medium containing 740 μM adenosine but no guanosine. This culture was divided into two equal parts, and one received 1 mM (final concentration) guanosine. At different times, samples were removed from both cultures, as described above, and enzyme activities in the cell extracts were determined. As Fig. 2 shows, the basal (uninduced) levels of enzyme activity were slightly higher than those in the standard strain (Fig. 1). The time after guanosine removal at which alpha-ketoglutarate dehydrogenase activity started increasing fluctuated somewhat (between 30 and 60 min), presumably depending on the amount of guanosine which had been carried over to the fresh medium with the cells.

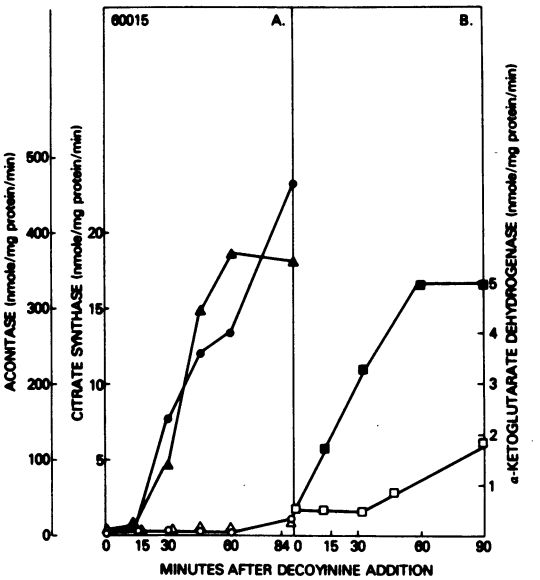

FIG. 1. *Effect of decoyinine on the specific activities of citrate synthase and aconitase (A) and on the specific activity of alpha-ketoglutarate dehydrogenase (B). Cells of the standard strain 60015 were grown until the OD_{600} reached 0.5. The culture was then divided into two parts; decoyinine was added to one part, and not to the other. Enzyme activities were determined at the times indicated on the figure. The enzyme activities of the decoyinine-treated culture are represented by the solid symbols, and those of the untreated culture are represented by the open symbols. Symbols: ▲ and △, citrate synthase; ● and ○, aconitase; ■ and □, alpha-ketoglutarate dehydrogenase.*

Induction of alpha-ketoglutarate dehydrogenase by acetate addition and in citrate cycle mutants. In previous investigations we found that adding acetate induced the synthesis of alpha-ketoglutarate dehydrogenase. This prompted us to investigate, by using mutants, which acetate derivative caused this induction. Table 2 shows effect of acetate on enzyme synthesis in standard strain 60015. The cells were grown in synthetic medium to an OD_{600} of 0.15, and then the culture was divided into two parts. To one part 20 mM (final concentration) potassium acetate (pH 7) was added, and the other part received the same amount of water. When the OD_{600} was 0.7, the cells of both cultures were harvested; crude extracts were prepared and used to determine the specific activities of the three enzymes. The presence of acetate caused a 30-fold increase in the specific

activity of alpha-ketoglutarate dehydrogenase. This increase was even more pronounced than the ninefold increase caused by decoyinine (Table 2). To determine whether the acetate derivative responsible for the induction of alpha-ketoglutarate dehydrogenase was a component of the citric acid cycle, we measured the activity of alpha-ketoglutarate dehydrogenase in two mutants lacking citrate synthase activity. Both strain 60871 (deficient in both aconitase and citrate synthase) and strain 61861 (deficient in citrate synthase) were grown in synthetic medium to an OD_{600} of 0.5, the cells were harvested and extracted, and the enzyme activities were determined. Both mutants showed a higher activity for alpha-ketoglutarate dehydrogenase than the standard strain, even without acetate addition. These results indicated that acetyl-CoA (or a derivative of acetyl-CoA which was not a component of the citric acid cycle) induced alpha-ketoglutarate dehydrogenase.

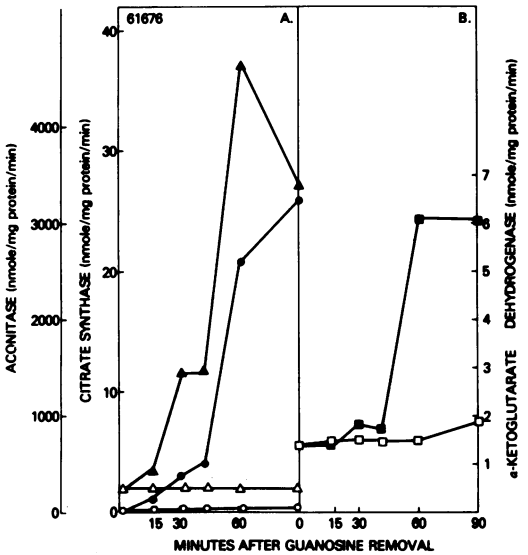

FIG. 2. *Specific activities of citrate synthase and aconitase (A) and alpha-ketoglutarate dehydrogenase (B) in a guanine auxotroph in the presence and absence of guanosine. The guanine-requiring strain was grown in synthetic medium containing 720 μM adenosine and 1 mM guanosine to an OD_{600} of 0.5, and then 20 ml was taken for enzyme assays. The cells of the remaining culture were collected by membrane filtration, suspended in medium containing no guanosine, and divided into two parts; only one of these parts received 1 mM guanosine. Enzyme activities were determined at the times indicated on the figure. The solid symbols represent the enzyme activities in the guanosine-limited culture, and the open symbols represent the enzyme activities in the culture supplemented with guanosine. Symbols: ▲ and △, citrate synthase; ● and ○, aconitase; ■ and □, alpha-ketoglutarate dehydrogenase.*

Determination of the intracellular concentrations of acetyl-CoA, alpha-ketoglutarate, and pyruvate after decoyinine addition or guanosine limitation. Because an increase in acetyl-CoA concentration could induce alpha-ketoglutarate dehydrogenase, we wanted to determine whether the increase in the specific activity of this enzyme during sporulation induction was correlated with an increase in acetyl-CoA concentration. Therefore, we developed a thin-layer chromatographic technique to measure the concentrations of ^{35}S-labeled acetyl-CoA and other CoA derivatives. Figure 3 shows a typical chromatogram of a ^{35}S-labeled cell extract. To analyze the effect of decoyinine, we grew strain 60005 (a prototroph) in synthetic medium which contained (from an OD_{600} of 0.1 on) 200 μCi of $Na_2{}^{35}SO_4$ per ml. When the OD_{600} was 0.5, the culture was divided into two equal parts, one of which received 1.44 mM decoyinine. Before the division and at different times thereafter, the cells of 0.75-ml portions of these cultures were extracted by using formic acid, and the intracellular concentrations of acetyl-CoA were determined. As Fig. 4 shows, the presence of decoyinine caused a significant increase in the

TABLE 2. *Enzyme activities of B. subtilis strains*[a]

Strain	Relevant defect(s)	Medium	Citrate synthase activity		Aconitase activity		α-Ketoglutarate dehydrogenase activity	
			−DEC	+DEC	−DEC	+DEC	−DEC	+DEC
60015	None	S	<1.3	18.0	<5.0	472	0.5	4.70
60015	None	S + acetate	<1.3		15.1		14.6	
60871	CTS, ACN		<1.3	1.3	6.78	4	19.6	25.3
61861	CTS		<1.3	<1.3	15.6	221	4.0	10.2
61461	MDH		<1.3	2.06	37.2	680	2.0	7.89
61423	Fumarase		5.50	42.7	7.3	441	0.8	9.14

[a] Abbreviations: ACN, aconitase; CTS, citrate synthase; MDH, malate dehydrogenase (EC 1.1.1.37); S, synthetic medium; DEC, decoyinine treatment. Enzyme activities are expressed in nanomoles per minute per milligram of protein.

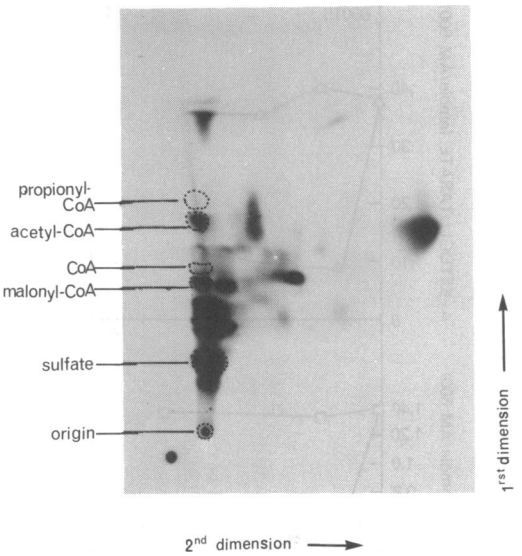

FIG. 3. *Two-dimensional separation of ^{35}S-labeled compounds in formic acid extracts of B. subtilis. Strain 60005 was grown in the presence of 0.05 μM $Na_2{}^{35}SO_4$ (200 μCi/ml) from an OD_{600} of 0.1 to an OD_{600} of 0.5. A formic acid extract of 0.4 AM_{600} unit was prepared (100 μl), and 5 μl was applied together with 10 μg of standards (indicated by circled spots) to a cellulose thin-layer plate. For the separation procedure, see text.*

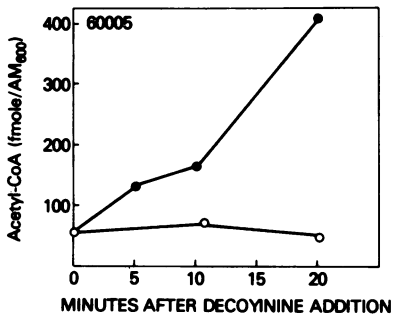

FIG. 4. *Effect of decoyinine on the concentration of acetyl-CoA. Strain 60005 (prototroph; labeled with 0.05 μM $Na_2{}^{35}SO_4$) was grown to an OD_{600} of 0.5, and the culture was divided into two parts; one of these parts received 1.44 mM decoyinine. Samples (0.75 ml) were taken just before and at different times after the division of the culture for extraction by formic acid; 5-μl portions of the extract were exposed to two-dimensional chromatography (Fig. 3), and the radioactivity in the acetyl-CoA spots was used to measure the amount of acetyl-CoA. Symbols: ●, decoyinine-treated culture; ○, untreated culture.*

intracellular concentration of acetyl-CoA, whereas the concentration of this compound remained constant in the control culture. We also used the guanine auxotroph 61676 (*purH guaA*) to perform the equivalent experiment under guanosine deprivation. We grew this strain initially in synthetic medium containing 1 mM guanosine and 200 μCi of $Na_2{}^{35}SO_4$ per ml. When the OD_{600} was 0.5, the cells were collected on a Millipore filter and suspended in the same volume of synthetic medium containing $Na_2{}^{35}SO_4$ (200 μCi/ml). Only one-half of this suspension received guanosine (1 mM). At different times thereafter, we took samples to determine the intracellular concentration of acetyl-CoA. As Fig. 5 shows, guanosine deprivation of the guanine auxotroph also caused an increase in acetyl-CoA concentration, but the effect was more delayed (and transient).

We also measured the intracellular concentration of acetyl-CoA in the citrate synthase mutant strain 60871, which had a high alpha-ketoglutarate dehydrogenase activity (Table 2). We grew this strain in synthetic medium containing 0.05 μM $Na_2{}^{35}SO_4$ (200 μCi/ml) until the OD_{600} was 0.5. Then, we determined the acetyl-CoA concentration as described above for three samples taken at 2-min intervals; this concentration was 227 fmol/AM_{600} unit, about four times higher than in the standard strain.

Whereas the addition of decoyinine to a culture of strain 60015 caused an increase in the acetyl-CoA concentration, it caused a decrease in the intracellular concentrations of alpha-ketoglutarate and pyruvate (Fig. 6).

Specific activities of citrate synthase and aconitase in various mutants. To analyze the mechanisms controlling the synthesis of citrate synthase and aconitase, we determined the specific activities of these enzymes in various mutants lacking the activities of the citrate cycle and of related enzymes after growth with and without decoyinine (Table 2). We inoculated each strain into 50 ml of synthetic medium (without aspartate where indicated) at an OD_{600} of 0.05 and grew the cultures to OD_{600} of 0.5, at which point we collected the cells from 20 ml of each culture for enzyme determinations. To the remaining cultures, we added 1.44 mM decoyinine and after 90 min again collected cells for enzyme measurements.

Citrate synthase had the highest specific activity in a fumarase mutant (strain 61423); the addition of decoyinine increased the activity further (Table 2). In contrast, no activity was detected in a malate dehydrogenase mutant (strain 61461), and addition of decoyinine caused almost no increase. Because these cells were grown in glucose, oxaloacetate (produced by pyruvate carboxylase) was the entry point of carbon compounds into the citrate cycle. As the equilibrium of malate dehydrogenase greatly favors malate,

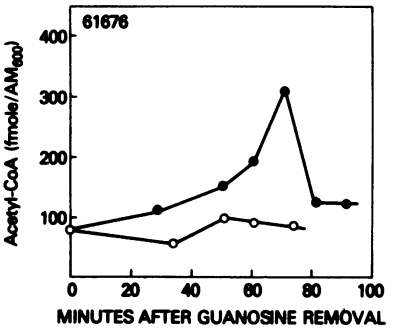

FIG. 5. *Effect of guanine limitation on the concentration of acetyl-CoA. Strain 61676 was grown in synthetic medium containing 20 μM adenosine, 1 mM guanosine, and 0.05 μM $Na_2{}^{35}SO_4$. When the culture reached an OD_{600} of 0.5, the cells were collected by filtration, suspended in the same volume of the same prewarmed medium containing no guanosine, and divided into two parts; only one of these parts received 1 mM guanosine. Symbols: ●, acetyl-CoA concentration in the culture without guanosine; ○, acetyl-CoA concentration in the culture containing 1 mM guanosine.*

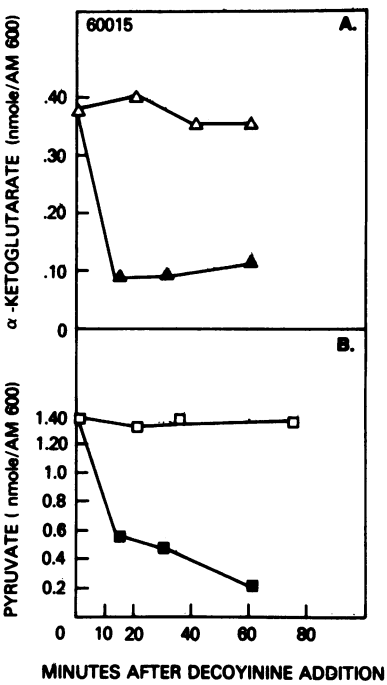

FIG. 6. *Effect of decoyinine on the concentrations of alpha-ketoglutarate (A) and pyruvate (B). Strain 60015 was grown in synthetic medium to an OD_{600} of 0.5; the culture was divided into two parts, one of which received 1.44 mM decoyinine. At different times samples were removed, and cells were extracted by formic acid treatment. Symbols: ▲ and ■, concentrations of metabolites in the decoyinine-treated culture; △ and □, concentrations of metabolites in the control culture. △, ▲, α-Ketoglutarate; □, ■, pyruvate.*

cells should normally contain more malate than oxaloacetate. However, for actively growing cells it is not possible to predict whether the concentration of malate is low in a fumarase mutant and high in a malate dehydrogenase mutant, as would be expected from the normal functioning of the cycle, or whether the opposite is the case, as would be expected when oxaloacetate is efficiently reduced to malate, fumarate, and succinate.

We tried to determine whether malate induced or repressed citrate synthase by adding malate to the cultures. Unfortunately, malate induced the malic enzyme, which interfered with the citrate synthase assay. However, when we measured the concentration of malate in the mutants described above, we found no malate in the fumarase mutant, whereas the malate dehydrogenase mutant contained more malate than the standard strain (Table 3). Therefore, malate apparently represses citrate synthase. Accordingly, when GMP synthesis was limited in standard strain 60015 by the addition of decoyinine, the intracellular concentration of malate decreased (Table 3), whereas the citrate synthase specific activity increased (Table 2).

TABLE 3. *Concentration of malate in different strains*

Strain	Relevant defect	Malate concn (nmol/AM_{600} unit)	
		Without decoyinine	With decoyinine
60015	None	0.0960	<0.0270
61461	Malate dehydrogenase	0.1730	0.0670
61423	Fumarase	<0.0020	<0.0013

DISCUSSION

When we decreased the rate at which guanine nucleotides were synthesized (by decoyinine addition or guanosine deprivation of a guanine auxotroph), a condition which induces sporulation, the specific activities of three enzymes of the citrate cycle increased. The following arguments suggest that the induction of these enzymes can be explained by changes in the concentrations of related metabolites.

The specific activity of alpha-ketoglutarate dehydrogenase also increased after acetate was added to a culture of the standard strain and was higher than normal in strains lacking citrate synthase activity (Table 2). Because the concentration of acetyl-CoA increased inside the cells in such mutants (shown for strain 60871) and

presumably in the standard strain after acetate was added, acetyl-CoA or a compound derived from it (but not a citrate cycle component) apparently induces alpha-ketoglutarate dehydrogenase. After the limitation of guanine nucleotides, the intracellular concentration of acetyl-CoA increased (Fig. 4) to levels normally observed in a citrate synthase mutant (strain 60871). Acetyl-CoA accumulated presumably because it was used less rapidly during the reduced growth. The higher acetyl-CoA concentration explains the increase in alpha-ketoglutarate dehydrogenase activity.

The regulation of aconitase has been investigated in several laboratories (9, 12, 19). The synthesis of this enzyme is induced by the addition of citrate to a culture of the standard strain and by intracellularly accumulated citrate in isocitrate dehydrogenase-deficient mutants (1, 4, 5, 19). The specific activity of aconitase is lower than normal in mutants lacking alpha-ketoglutarate dehydrogenase (1, 5, 21) or in the presence of glutamate in strains (such as the standard strain) in which glutamate can be converted to alpha-ketoglutarate (10). Therefore, the inducing effect of citrate is counteracted by the repressing effect of alpha-ketoglutarate (glutamate in the medium) (19).

After the addition of decoyinine, we found that the specific activity of aconitase increased drastically, whereas the intracellular concentration of alpha-ketoglutarate decreased by 70% (Fig. 6) (the concentration of citrate remained undetectable [less than 0.08 nmol/AM_{600} unit] when we attempted to measure it enzymatically). If the citrate synthase-deficient strain 61861 was exposed to decoyinine, the specific activity of aconitase increased only half as much as in the standard strain (Table 2). These observations suggest that aconitase activity increased, after decoyinine was added, in part because the concentration of alpha-ketoglutarate decreased and in part because the concentration of citrate increased.

Like aconitase, citrate synthase is repressed by glutamate (3) via alpha-ketoglutarate (10). The following evidence indicates that this enzyme is also repressed by malate. In the standard strain, the addition of decoyinine reduced the intracellular concentration of malate (Table 3) and increased the specific activity of citrate synthase (Table 2). The latter was high in the fumarase-deficient strain 61423 (Table 2), which contained less malate than the standard strain (Table 3), and low in the malate dehydrogenase-deficient strain 61461 (Table 2), which contained more malate than the standard strain (Table 3). After GMP synthesis was limited, the specific activity of citrate synthase increased drastically, whereas the intracellular concentrations of alpha-ketoglutarate and malate decreased.

A limitation of adenine also causes an increase in the specific activities of citrate synthase and aconitase (2). Because this limitation should decrease not only adenine nucleotides but also, indirectly, guanine nucleotides (16), we believe that the observed increases in enzyme specific activities also resulted from changes in the concentrations of citrate cycle intermediates.

In conclusion, our results have demonstrated that the sporulation-related increases in the specific activities of citrate synthase, aconitase, and alpha-ketoglutarate dehydrogenase can be explained by changes in the concentrations of metabolites, such as acetyl-CoA, alpha-ketoglutarate, and malate.

The increases in the specific activities of these enzymes are probably necessary for regeneration of ATP if sporulation is caused by the limitation of rapidly metabolizable carbon sources; mutants deficient in any one of these enzyme activities cannot sporulate under these conditions (5). However, these enzymes are not needed when sporulation is induced by adding decoyinine (i.e., GMP deprivation) to a glucose-containing medium, because ATP can then be regenerated by glycolysis.

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