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Isolation and Characterization of Tricarboxylic Acid Cycle Mutants of *Bacillus subtilis*

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A technique was developed for the detection, on agar, of mutants of *Bacillus subtilis* that lacked a functional tricarboxylic acid cycle. Mutants devoid of detectable levels of aconitase, isocitric dehydrogenase, alpha-ketoglutarate dehydrogenase, succinic dehydrogenase, fumarase, and malate dehydrogenase have been isolated and characterized. Several mutants with conditionally expressible lesions, including a mutant with a heat-sensitive citrate synthase, have also been isolated. All of the mutants examined express all the biochemical markers normally absent in early-stage sporulation mutants except elastase, and some of these mutants sporulated nearly as well as the prototroph.

Mutants of *Bacillus subtilis* that lack an enzyme of the tricarboxylic acid cycle have been observed to be oligosporogenic, i.e., the probability of a cell to form a mature spore was found to be considerably less than that of a cell of the parent strain (3-6, 12, 13). In most cases described, these mutants were isolated on the basis of their unusual colonial morphology and pigmentation. The colonial morphology was in part determined by the inability of the cells to complete sporulation (3-5). These mutants were subsequently observed to have lesions in the tricarboxylic acid cycle. Therefore, in many cases, the method of isolation may have precluded the selection of sporogenic mutants which lacked a functional tricarboxylic acid cycle. The purpose of this study was to develop a technique for the isolation of mutants that directly tested for a functional tricarboxylic acid cycle. With these mutants, one could reexamine the relationship between the presence of enzymes of the tricarboxylic acid cycle and sporulation. Mutants which lacked succinic dehydrogenase, produced low levels of proteinase, and had low antibiotic activity against *Staphylococcus aureus* (4). Some other mutants isolated by Freese and co-workers (5), with a lesion in the tricarboxylic acid cycle, lacked either antibiotic or proteinase activities. All the tricarboxylic acid cycle mutants isolated in this study showed normal activities of other early-stage sporulation events.

MATERIALS AND METHODS

Organism. *B. subtilis* Marburg 168, auxotrophic for indole or tryptophan, and derivatives of this strain were used throughout this study. *B. subtilis* strain H (obtained from J. Spizizen, Scripps Clinic and Research Foundation, LaJolla, Calif.) and *S. aureus* UW209 were used to test for antibiotic production.

The medium used for the isolation of mutants contained the following (in 1 liter of distilled water): nutrient broth (Difco), 8 g; bromocresol purple, 0.015 g; dibasic potassium phosphate, 0.5 g; and agar, 20 g. The *p*H was adjusted to 6.6 before sterilization. After autoclaving, the medium was cooled to 50 C, and sterilized solutions or suspensions of the following compounds were added: 20% $CaCO_3$, to give a final concentration of 1.0%; sufficient sterile 50% glucose to give a final concentration of 0.2%; 5 ml of a metal mixture containing 0.14 M CaCl, 0.01 M $MnCl_2$, and 0.20 M $MgCl_2$; and 0.5 ml of filter-sterilized $FeCl_3$ (2×10^{-3} M in 0.01 N HCl).

The purification medium used for the reisolation and testing of all mutants before use in experiments contained the following (in 1 liter of distilled water): nutrient broth, 8 g; bromocresol purple, 0.015 g; and agar, 20 g. The *p*H was adjusted to 6.0 before autoclaving. After cooling to 50 C, 5 ml of the metal mixture indicated above and sufficient sterile glucose solution to give a final concentration of 0.1% were added. Mutants lacking a functional tricarboxylic acid cycle form yellow colonies after 24 hr of incubation, whereas the wild-type colonies turn the medium blue after 24 hr at 37 C. It is possible to detect wild-type contaminants representing less than 1% of the population on this medium.

The mutants were routinely stored in vials containing a medium of the following composition: nutrient broth, 8 g; glucose, 1 g; potassium bicarbonate, 1 g; and agar, 17 g. The *p*H was adjusted to 7.5 before sterilization.

The minimal phosphate medium contained (in 1 liter of distilled water): $(NH_4)_2SO_4$, 2 g; K_2HPO_4, 6 g; KH_2PO_4, 14 g; and ethylenediaminetetracetic acid

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(EDTA), 0.1 g. The *p*H was adjusted to 7.0 and the solution was autoclaved. After the medium had cooled, 1 ml of the metal mixture indicated above was added. The minimal Tris medium contained (in 1 liter of distilled water): tris(hydroxymethyl)aminomethane (Tris), 1.2 g; KH_2PO_4, 0.136 g; $MnCl_2$, 0.33 g; NH_4Cl, 0.8 g; KCl, 5 g; and Na_2SO_4, 0.014 g. The *p*H was adjusted to 7.0 before autoclaving. The nutrient sporulation medium (NSM) contained 8 g of nutrient broth per liter. After autoclaving, 1 ml of the metal mixture indicated above was added.

Mutagenesis. Approximately 100 mg of dry spores was transferred to a sterile test tube and placed in a vacuum oven. After evacuation of the oven, the temperature was slowly increased to 105 C and the spores were heated for 9 hr as described by Northrop and Slepecky (10). The treated spores were then plated for isolation of mutants or stored in a dessicator for future use.

Growth of cultures and preparation of extracts. Spores of *B. subtilis* Marburg 168 were grown in NSM. A loopful of a 24-hr culture grown at 37 C on NSM plus 2.0% agar was transferred to a 500-ml flask containing 50 ml of medium, and the cultures were incubated at 37 C on a rotary shaker until visible growth had occurred. The contents of one flask were transferred to 400 ml of NSM in a 2-liter flask. The culture was incubated on a rotary shaker for 48 hr and centrifuged for 10 min at 10,400 × *g* to collect the spores. The spores from several cultures were pooled, washed 12 times with a buffer containing 10^{-3} M Tris, 10^{-3} M EDTA, 10^{-3} M citric acid, and 0.1 M KCl (*p*H 7.5). The washed spores were lyophilized and stored in a dessicator at 4 C.

Cultures to be used for enzyme assays were streaked on purification medium and incubated for 24 hr at 37 C, and an inoculum from a yellow colony was transferred to a plate of blood-agar base. After 12 hr at 37 C, an isolated colony was used to inoculate 50 ml of the growth medium in a 500-ml flask. The culture was incubated at 37 C for 2 hr, and the contents were transferred to 400 ml of the growth medium in a 2-liter flask. These cultures were incubated at 37 C and were harvested 2 to 3 hr after the end of exponential growth by centrifuging at 10,400 × *g* for 10 min. The purity of the cultures was routinely checked by streaking a loopful from each flask onto purification medium. The plates were incubated for 24 hr at 37 C, and only those cells from flasks which did not yield any blue color in the agar were used for enzyme assays. The cells were washed twice in ice-cold buffer containing 0.1 M Tris, 10^{-3} M EDTA, and 10^{-3} M citric acid (*p*H 7.5) and were stored frozen at −20 C. The frozen cells were thawed in 5 ml of the same buffer and were broken by two passes through a French pressure cell at 4 C. The extract was centrifuged at 4 C for 10 min at 5,100 × *g*, and the supernatant fraction was used for enzyme assays.

Enzymatic assays. The following enzymes were assayed as previously described (2, 7): citrate synthase [citrate oxalacetate-lyase (CoA acetylating), EC 4.1.3.7]; aconitase [citrate (isocitrate) hydro-lyase, EC 4.2.1.3]; isocitric dehydrogenase (NADP) (*threo*-D_s-isocitrate:NADP oxidoreductase, EC 1.1.1.42); succinic dehydrogenase [succinate:(acceptor) oxidoreductase, EC 1.3.99.1], fumarase (L-malate hydro-lyase, EC 4.2.1.2); and malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37). The reaction mixture for the assay of α-ketoglutarate dehydrogenase contained: potassium phosphate buffer (*p*H 7.0), 50 mM; coenzyme A, 0.1 mM; nicotinamide adenine dinucleotide, 2 mM; cysteine, 3 mM; magnesium chloride, 1 mM; thiamine pyrophosphate, 0.2 mM; α-ketoglutarate, 5 mM; quinacrine hydrochloride, 0.1 mM; and sufficient extract to cause a change in absorbance at 340 nm of approximately 0.03 per min. The reaction was monitored with a Gilford model 2000 recording spectrophotometer. Quinacrine hydrochloride was required to inhibit a soluble reduced nicotinamide adenine dinucleotide oxidase that interfered with this assay in the absence of the inhibitor.

Estimation of sporulation frequencies. The inoculum for each culture was prepared as described above. Samples of the culture for viable cell counts were taken 0.5 hr after exponential growth had ceased and were diluted in sterile phosphate buffer containing 14 g of K_2HPO_4 and 6 g of KH_2PO_4 (*p*H 7.0), in 1 liter of distilled water. Dilutions were plated on purification medium and incubated at 37 C for 24 to 48 hr. Samples for spore counts were taken at several times after exponential growth and were heated at 80 C for 10 min. After dilution in phosphate buffer, samples were plated on purification agar. This method provided a means for detecting wild-type revertants in the culture. If revertants were detected, the experiment was discarded. Sporulation frequencies (S/V) are reported as the thermostable colony-forming units (S) divided by the viable count (V).

RESULTS

Isolation and storage of mutants. When *B. subtilis* 168 was grown in nutrient broth supplemented with glucose, the *p*H dropped from 7.0 to approximately 5.5 because of the accumulation of organic acids. After exhaustion of glucose, the synthesis of enzymes of the tricarboxylic acid cycle was derepressed and the *p*H increased (6, 8). The *p*H drop was greater when tricarboxylic acid cycle mutants were grown in media containing small amounts of glucose, and the *p*H of the medium did not increase after glucose was exhausted. Tricarboxylic acid cycle mutants grown on the isolation agar medium described in Materials and Methods formed halos around colonies (resulting from solubilization of $CaCO_3$ by organic acids accumulated by the mutants.) In preliminary tests with mixtures of an aconitase-negative mutant and *B. subtilis* 168, it was found that a single clone of an aconitase-negative mutant could be detected on a plate containing 100 or more clones of the parent strain. Many of the tricarboxylic acid cycle mutants isolated by spreading treated spores on this medium were unstable and appeared to revert at high frequencies when first isolated. After 10 or more reisolations at 12-hr intervals, the isolates were stabi-

lized. The mutants rapidly lost viability on media containing glucose or on nutrient agar alone and were stored on nutrient agar slants containing glucose and calcium carbonate. Cultures lost viability after 2 weeks on this medium and were lyophilized or were stored in 5% dimethyl sulfoxide in liquid nitrogen for long-term preservation.

Dissimulation of ^{14}C-succinate and ^{14}C-glutamate. After isolation and stabilization, all mutants were characterized by a modification of the technique described by Fortnagel and Freese (3) and subsequently used by Rutberg and Hoch (11). ^{14}C-succinate was used instead of ^{14}C-glutamate because it gave a more easily interpretable pattern of labeling of tricarboxylic acid cycle intermediates with our strains. ^{14}C-glutamate labels other intermediates by reversal of the first half of the tricarboxylic acid cycle. Because the reaction catalazed by α-ketoglutarate dehydrogenase is irreversible, ^{14}C-succinate pulses provide more readily interpretable labeling patterns. Cultures were incubated with ^{14}C-succinate, and the extracted pools were analyzed by thin-layer chromatography and autoradiography (3). Mutants lacking α-ketoglutarate dehydrogenase were distinguishable from the parent strain only after labeling with ^{14}C-glutamate. Aconitase and isocitrate dehydrogenase mutants accumulated asparate and citrate (or isocitrate) but did not label glutamate or α-ketoglutarate. The single α-ketoglutarate dehydrogenase mutant studied accumulated ^{14}C-glutamate and ^{14}C-α-ketoglutarate but no ^{14}C-citrate when labeled with ^{14}C-succinate. Only ^{14}C-α-ketoglutarate, ^{14}C-glutamate, and ^{14}C-citrate (or isocitrate) accumulated when this mutant was incubated with ^{14}C-glutamate. Succinate dehydrogenase-negative mutants accumulated only ^{14}C-succinate when incubated with ^{14}C-succinate. Fumarase mutants accumulated fumarate; no succinate was observed in cell pools. The single malate dehydrogenase mutant accumulated ^{14}C-glutamate, ^{14}C-malate, and ^{14}C-succinate; surprisingly, ^{14}C-fumarate and other intermediates were not detected. The accumulation of glutamate by this mutant is perhaps due to the conversion of malate, via malic enzyme to pyruvate and the reentry of radioactive carbon into the tricarboxylic acid cycle as ^{14}C-acetate.

One mutant, apparently deficient in citrate synthase (HS1A17), and two which appeared to lack both citrate synthase and aconitase (HS1A14 and HS2A1) when grown on NSM (Table 1) and were glutamate auxotrophs (Table 2) accumulated very small amounts of citrate (or isocitrate). This finding prompted further studies which have shown them to be leaky mutants or mutants with a modified enzyme.

Nutritional studies. Mutants deficient in citrate synthase, aconitase, and isocitric dehydrogenase would be expected to be glutamate auxotrophs (3, 6, 11), and this assumption was borne out in further tests of the mutants isolated (see Table 2). Mutants with lesions in α-ketoglutarate dehydrogenase, succinate dehydrogenase, and fumarase cannot grow on media containing glutamate as the sole energy source. A single mutant lacking malate dehydrogenase was capable of growing on glutamate as the sole energy source. All mutants were tested for a tryptophan requirement to insure that they were derived from *B. subtilis* 168.

Some mutants that were isolated as acid-accumulating mutants and were subsequently shown to be devoid of citrate synthase (HS1A17), aconitase (HS1A12), and isocitric dehydrogenase (HS1A15) when grown on NSM (Table 1) were able to grow on a synthetic glucose medium devoid of glutamate, arginine, and proline at 37 C (Table 2). The growth of these mutants on a minimal glucose medium was shown to be temperature dependent (Table 3). The parent strain, *B. subtilis* 168, grew on a minimal glucose-agar medium at 37 and 50 C, whereas none of the mutants listed above grew at 50 C on this medium. Mutant HS1A17 grew at 50 C when glutamate or citrate was added to the synthetic medium containing glucose, and all were capable of growing on glutamate as an energy source at 50 C. These results indicated that a modified enzyme in the first half of the tricarboxylic acid cycle was inactivated during growth at 50 C and that this defect resulted in a temperature-dependent requirement for citrate or glutamate. These mutants formed low levels (compared to *B. subtilis* 168) of enzymes when grown on heart infusion broth at 37 C and accumulated organic acids on purification medium agar.

Mutant HS1A17 has been studied in some detail. The growth rate of this mutant on a synthetic medium containing glucose plus 15 amino acids (*see* Table 2) decreases at temperatures above 45 C and it does not grow above 47.5 C on this medium. When the medium is supplemented with arginine or proline and glutamate, the growth rate is identical to that of the wild type at 50 C (D. Johnson, R. Carls, and R. Hanson, *unpublished data*). Citrate synthase was partially purified from extracts of cells grown on a minimal succinate and heart infusion broth, and the enzyme was found to be inactivated by 5 min of exposure to 44 C, whereas the wild-type enzyme was stable at the same protein concentration at 46 to 48 C (Fig. 1). The K_m values for oxalacetate and acetyl-coenzyme A were found to be identical to those of the wild-type enzyme (2), but the citrate synthase from HS1A17 was not

TABLE 1. *Specific activities of citric acid cycle enzymes in Bacillus subtilis 168 and tricarboxylic acid cycle mutants in extracts of cells grown in nutrient sporulation medium*

Source of enzymes	Specific activity[a]						
	CS[b]	Acon	IDH	AKGDH	SDH	Fum	MDH
B. subtilis 168	3.5	2.5	9.0	0.15	10.0	7.0	40
B. subtilis HS1A17	<0.05	0.3	9.0	—[c]	7.9	6.5	30
B. subtilis HS1A14	<0.05	<0.10	4.0	—	8.0	1.5	36
B. subtilis HS2A1	<0.05	<0.10	11.5	—	7.3	5.6	44
B. subtilis HS1A3	3.6	<0.10	6.0	—	—	6.7	—
B. subtilis HS1A11	4.4	<0.10	13.3	0.1	7.9	5.0	42
B. subtilis HS1A12	3.3	<0.10	8.6	—	—	11.5	—
B. subtilis HS1A19	1.8	<0.10	6.8	—	6.0	3.2	59
B. subtilis HS1A23	0.3	<0.10	14.7	—	—	5.4	—
B. subtilis HS3A1	2.3	<0.10	6.9	—	7.7	1.2	21.7
B. subtilis HS3A20	0.4	<0.10	59.0	—	7.1	5.8	91
B. subtilis HS1A15	3.9	<0.10	<0.01	—	6.9	6.6	27
B. subtilis HS2A2	13.0	15.0	<0.01	—	6.3	7.0	55
B. subtilis HS3A16	3.9	0.3	6.7	<0.01	16.0	7.9	23
B. subtilis HS3A17	2.7	0.6	7.5	0.04	56.5	5.2	19
B. subtilis HS1A1	2.9	0.2	6.6	2.0	<1.0	14.3	—
B. subtilis HS1A7	2.8	0.3	4.1	—	<1.0	4.7	12
B. subtilis HS1A9	0.6	0.5	3.4	—	<1.0	1.9	21
B. subtilis HS1A13	2.0	1.0	11.7	—	<1.0	3.1	9.7
B. subtilis HS1A16	2.3	0.9	7.3	—	<1.0	5.0	16.5
B. subtilis HS1A18	1.3	0.3	4.5	—	<1.0	1.9	12.7
B. subtilis HS1A22	1.9	0.5	5.0	—	<1.0	6.9	32.0
B. subtilis HS1A6	1.5	0.8	6.1	—	—	<0.1	—
B. subtilis HS1A8	2.2	0.7	4.8	—	—	<0.1	—
B. subtilis HS1A21	2.1	0.4	5.0	—	10.7	3.6	<1.0

[a] Specific activities are expressed in micromoles per minute per milligram of protein multiplied by 10.

[b] Enzymes assayed were citrate synthase (CS), aconitase (Acon), isocitrate dehydrogenase (IDH), α-ketoglutarate dehydrogenase (ADGDH), succinate dehydrogenase (SDH), fumarase (Fum), and malate dehydrogenase (MDH).

[c] Indicates that the levels of this enzyme were not determined.

significantly inhibited by an adenosine triphosphate (ATP) concentration that caused 80% inhibition of the wild-type enzyme (Table 4). Mutants HS1A12 and HS1A15 with apparent temperature-sensitive defects in aconitase and isocitric dehydrogenase were not characterized further.

One mutant (HS1A8) devoid of fumarase and one devoid of malate dehydrogenase (HS1A21) also appeared to have conditionally expressed defects in glutamate synthesis (Table 3). This phenomenon was not investigated further. One possible explanation is that the intracellular accumulation of dicarboxylic acids results in sensitization of one or more of the enzymes in the first half of the tricarboxylic acid cycle to higher temperatures. An aconitase mutant, HS1A12, also shows a conditionally expressible defect in the second half of the tricarboxylic acid cycle because it does not grow on glutamate as a sole energy source at 50 C.

All other mutants were tested for growth on several media at temperatures between 25 and 50 C and none exhibited conditionally expressible phenotypes.

Enzymatic assays. Table 1 shows the results of enzymatic assays of mutants grown on NSM. Because a few mutants that were defective for an enzyme gave autoradiograms that indicated that they were leaky, attempts were made to obtain detectable levels of enzymatic activity by growth on several media. Mutant HS1A17, with a temperature-sensitive citrate synthase, had undetectable levels of enzyme on NSM but formed 10% of the wild-type levels of citrate synthase on heart infusion broth and formed detectable levels of citrate synthase in defined medium with glucose or succinate as the energy source. Other mutants devoid of citrate synthase and aconitase when grown on NSM (HS1A14 and HS2A1) also formed detectable levels of enzyme when grown on heart infusion broth. These results were predictable from the autoradiographic studies described above.

Mutant HS1A12, an aconitase mutant which synthesized small amounts of ^{14}C-glutamate from ^{14}C-succinate, synthesized detectable levels of aconitase in heart infusion broth but not in NSM. All other mutants were tested for ability

TABLE 2. *Nutritional requirements of tricarboxylic acid (TCA) cycle mutants of Bacillus subtilis*

Organism	TCA cycle lesion[a]	Media[b]			
		glc	try-glc	try-glu	try-glc-glu
B. subtilis 168		–[c]	+	+	+
B. subtilis HS1A14	*cts-acn*	–	–	+	+
B. subtilis HS2A1	*cts-acn*	–	–	+	+
B. subtilis HS1A17	*cts*	–	+	+	+
B. subtilis HS1A3	*acn*	–	–	+	+
B. subtilis HS1A11	*acn*	–	–	+	+
B. subtilis HS1A19	*acn*	–	–	+	+
B. subtilis HS1A23	*acn*	–	–	+	+
B. subtilis HS3A1	*acn*	–	–	+	+
B. subtilis HS3A20	*acn*	–	–	+	+
B. subtilis HS1A12	*acn*	–	+	+	+
B. subtilis HS2A2	*idh*	–	–	+	+
B. subtilis HS1A15	*idh*	–	+	+	+
B. subtilis HS3A16	*kdh*	–	+	–	+
B. subtilis HS3A17	Unknown[d]	–	+	–	+
B. subtilis HS1A1	*sdh*	–	+	–	+
B. subtilis HS1A7	*sdh*	–	+	–	+
B. subtilis HS1A9	*sdh*	–	+	–	+
B. subtilis HS1A13	*sdh*	–	+	–	+
B. subtilis HS1A16	*sdh*	–	+	–	+
B. subtilis HS1A22	*sdh*	–	+	–	+
B. subtilis HS1A6	*fum*	–	+	–	+
B. subtilis HS1A8	*fum*	–	+	–	+
B. subtilis HS1A21	*mdh*	–	+	+	+

[a] Determined by enzyme assay. Abbreviations: *cts*, citrate synthase; *acn*, aconitase; *idh*, isocitrate dehydrogenase; *kdh*, α-ketoglutarate dehydrogenase; *sdh*, succinic dehydrogenase; *fum*, fumarase; *mdh*, L-malate dehydrogenase. Superscript minus indicates the absence of an enzyme; no superscript indicates a leaky mutant with low but detectable levels of the enzyme indicated.

[b] Minimal Tris medium plates plus 15 amino acids plus additions indicated. The 15 L-amino acids added were (1 mg/ml each): alanine, asparagine, aspartate, cysteine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tyrosine, and valine. The amino acids were neutralized with $NaHCO_3$ and were filter sterilized. Abbreviations: try, tryptophan (10 μg/ml); glc, glucose (0.5%); glu, glutamate (50 mM).

[c] Growth (+); no growth (–).

[d] Succinyl-CoA synthetase was not assayed. Other characteristics of this organism indicated that the lesion might be in this enzyme.

to synthesize enzymes in synthetic media and heart infusion broth. Only one other mutant, HS1A15, a conditionally expressible isocitrate dehydrogenase mutant, synthesized detectable levels of enzyme in heart infusion broth but not NSM.

There was excellent agreement among the three methods for characterization of mutants. In each case that an incomplete block was detected, it was detectable by labeling patterns and enzymatic assays of extracts from cells grown on several media. In all other mutants, all three methods used provided data supporting a complete block at one reaction in the tricarboxylic acid cycle.

Several other interesting observations are worth brief mention. Mutant HS3A20, an aconitase-negative mutant, forms 9 to 10 times more isocitrate dehydrogenase than the wild-type strain on NSM. This phenomenon is possibly due to the inability of this strain to synthesize α-ketoglutarate, an effector of repression of citrate synthase, aconitase, and isocitrate dehydrogenase in *B. subtilis* (2). An isocitrate dehydrogenase mutant, HS2A2, forms a high level of citrate synthase and aconitase, possibly for the same reason. Other citrate synthase, aconitase, and isocitrate dehydrogenase mutants do not have derepressed levels of the remaining enzymes of the first half of the tricarboxylic acid cycle when grown under the same conditions. This is believed to be due to the fact that compounds that are metabolized to form α-ketoglutarate were not exhausted when these cultures were harvested (*unpublished data*). When grown under conditions of glucose limitation, all mutants with low or undetectable levels of citrate synthase and aconitase form very high levels of isocitrate dehydrogenase (Table 5.) It was possible to increase the specific activities of isocitrate dehydrogenase 28- to 130-fold over the wild-type levels in these mutants. Rutberg and Hoch (11) previously reported that a block in aconitase increases the specific activity of isocitric dehydrogenase and vice versa in cells grown on a modified NSM medium.

Mutants HS1A14 and HS2A1 that have low but detectable levels of citrate synthase when grown on heart infusion broth also have low levels of aconitase. It has been shown that the synthesis of citrate synthase and aconitase was coordinate in *B. subtilis* (2), and this phenomenon may reflect a mutation in a gene controlling the synthesis of both enzymes. Attempts to derepress further the synthesis of citrate synthase and aconitase by limitation of glucose in mutants HS1A14, HS1A17, and HS2A1 were partly successful (compare Tables 1 and 5). Although the enzyme levels obtained were low compared to the activities obtained with the wild-type strain, limitation of glucose resulted in considerably higher levels of the respective enzymes than have been obtained by growth on complex media.

Expression of early-stage sporulation events in tricarboxylic acid cycle mutants. Mutants that are unable to synthesize an enzyme that appears early in sporulation often do not express other

TABLE 3. *Growth of some tricarboxylic acid (TCA) cycle mutants on different media at 37 and 50 C*

Organism	Apparent TCA cycle lesion[a]	Media[b]							
		try glc		try glc-cit		try glu		try glc-glu	
		37 C	50 C	37 C	50 C	37 C	50 C	37 C	50 C
Bacillus subtilis 168	None	+	+	+	+	+	+	+	+
B. subtilis HS1A17	*cts*	+	−	+	+	+	+	+	+
B. subtilis HS1A11	*acn*	−	−	−	−	+	−	+	+
B. subtilis HS1A12	*acn*	+	−	+	+	+	−	+	+
B. subtilis HS1A15	*idh*	+	−	+	+	+	+	+	+
B. subtilis HS1A8	*fum*⁻	+	−	+	−	−	−	+	+
B. subtilis HS1A21	*mdh*⁻	+	−	+	+	+	+	+	+

[a] Symbols for the lesions as in Table 2.

[b] Minimal Tris medium plated plus 15 amino acids plus additions indicated. The additions to the minimal Tris medium are identical to those described in Table 2, except for citrate (5 g/liter); +, growth; −, no growth.

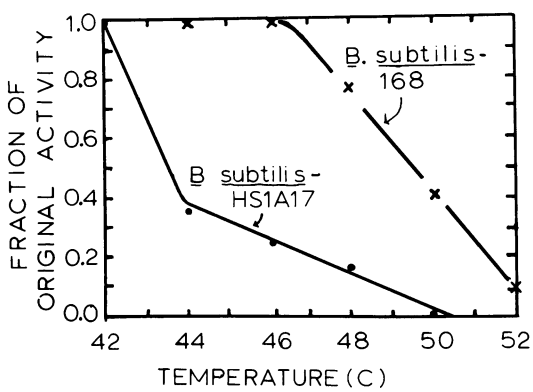

FIG. 1. *Stability of citrate synthases from Bacillus subtilis 168 and B. subtilis HS1A17 at different temperatures. Partially purified preparations of citrate synthase from B. subtilis 168 and B. subtilis HS1A17 were incubated for 5 min at the temperatures indicated in 10^{-3} M tris(hydroxymethyl)aminomethane, 10^{-2} M ethylenediaminetetracetic acid, and 10^{-3} M citric acid, pH 7.8. The optical density (280 nm) was adjusted to 18 prior to heating.*

TABLE 4. *Inhibition of partially purified citrate synthase from Bacillus subtilis 168 and B. subtilis HS1A17 by ATP*

Source of enzyme[a]	ATP added (mM)	Control activity[b] (%)
B. subtilis 168	None	100
	2	45
	4	19
	6	20
B. subtilis HS1A17	None	100
	2	123
	4	81
	6	83
	10	78

[a] The enzyme preparations were partially purified from extracts of *B. subtilis* 168 and *B. subtilis* HS1A17 by precipitation with ammonium sulfate (R. A. Carls, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1970).

[b] The reaction mixtures contained in 1.0 ml: 10 μmoles of tris(hydroxymethyl)aminomethane-acetate, *p*H 7.8; 1.0 μmole of oxaloacetate; 0.06 μmole of 5,5'-dithobis-(2-nitrobenzoic acid), and 0.1 μmole of acetyl coenzyme A and ATP as indicated.

sporulation-related events (1, 12). For this reason, it is often difficult to recognize the primary lesion in many asporogenous and oligosporogenous mutants (9). All the mutants isolated showed normal proteolytic activity against the following substrates that have been used by Balassa (1) for the isolation of different proteinase-negative mutants: gelatin, casein, denatured albumin, native albumin, protamine, and hemoglobin. All mutants showed antibacterial activity against *B. subtilis* strain H and *S. aureus* used as indicators for distinct antibacterial agents described by Shaeffer (12) and Balassa (1). None of the mutants reduced triphenyltetrazolium chloride when assayed by the method of Balassa (1) and none except HS1A21 (a malic dehydrogenase mutant) hydrolyzed elastin. The inability to hydrolyze elastin probably reflects the loss in viability of the cells on NSM agar. Elastase activity appears after 4 to 7 days of incubation at 37 C, and the mutants examined lose viability rapidly after 2 to 4 days of growth on this medium.

Sporulation of tricarboxylic acid cycle mutants. In contrast to previous results (3–6, 9, 12), some of the mutants isolated in this study sporulate at frequencies approaching those observed with the parent strain (Table 6). Fifty-seven per cent of the viable cells at $t_{0.5}$ of *B. subtilis* Marburg 168 form spores in a NSM medium. Mutants devoid of aconitase (HS1A12 and HS1A19) and succinic dehydrogenase (HS1A7, HS1A9, HS1A13, HS1A16, and HS1A1) formed spores in 1 to

TABLE 5. *Specific activities of citrate synthase, aconitase, and isocitric dehydrogenase in extracts of cells grown on a glucose-limited medium*[a]

Source of enzyme	Specific activity[b]		
	CS	Acon	IDH
Bacillus subtilis 168	3.0	11.0	10.0
B. subtilis HS1A17	0.2	1.5	430
B. subtilis HS1A14	0.9	ND[c]	1300
B. subtilis HS2A1	0.1	ND[c]	280

[a] The growth medium contained (per liter of medium): phosphate buffer (10 mM, *p*H 7.0), glucose (0.1%); glutamate, arginine, and proline (1 mM each), tryptophan (20 mg/ml); a mixture of the 15 amino acids listed in Table 2 (10 mg each); and 5 ml of the metal mixture described in Materials and Methods. Cells harvested 2 hr after the end of logarithmic growth and the enzymes were assayed as described in Materials and Methods.

[b] Specific activities are expressed in micromoles per minute per milligram of protein. Figures shown to be multiplied by 10^3. Enzymes assayed were: citrate synthase (CS), aconitase (Acon), and isocitrate dehydrogenase (IDH).

[c] Not detectable; less than 0.01 units per milligram of protein.

TABLE 6. *Sporulation of tricarboxylic acid (TCA) cycle mutants in nutrient sporulation medium (NSM) and on heart infusion broth (HIB)*

Organism	TCA cycle lesion[a]	S/V[b]	
		NSM	HIB
Bacillus subtilis 168		5.7×10^{-1}	5×10^{-2}
B. subtilis HS1A17	*cts*	6.7×10^{-1}	8×10^{-5}
B. subtilis HS1A14	*cts-acn*	1.6×10^{-2}	1.2×10^{-4}
B. subtilis HS2A1	*cts-acn*	7.3×10^{-3}	3.1×10^{-6}
B. subtilis HS1A3	*acn*	2.2×10^{-3}	$<10^{-7}$
B. subtilis HS1A11	*acn*	$<10^{-7}$	1.2×10^{-5}
B. subtilis HS1A12	*acn*	1.8×10^{-2}	1.7×10^{-1}
B. subtilis HS1A19	*acn*	1.9×10^{-2}	5.5×10^{-5}
B. subtilis HS1A23	*acn*	3.2×10^{-4}	$<10^{-7}$
B. subtilis HS3A1	*acn*	1.3×10^{-6}	5.5×10^{-5}
B. subtilis HS3A20	*acn*	5.2×10^{-3}	2.6×10^{-3}
B. subtilis HS1A15	*idh*	$<10^{-7}$	1.4×10^{-6}
B. subtilis HS2A2	*idh*	$<10^{-7}$	2.23×10^{-4}
B. subtilis HS3A16	*kdh*	$<10^{-7}$	$<10^{-7}$
B. subtilis HS3A17	*scs*	$<10^{-7}$	$<10^{-7}$
B. subtilis HS1A1	*sdh*	5.5×10^{-2}	8×10^{-2}
B. subtilis HS1A7	*sdh*	8.4×10^{-2}	2.6×10^{-2}
B. subtilis HS1A9	*sdh*	11×10^{-2}	1.3×10^{-2}
B. subtilis HS1A13	*sdh*	2.5×10^{-2}	2.9×10^{-2}
B. subtilis HS1A16	*sdh*	7.8×10^{-2}	1×10^{-1}
B. subtilis HS1A18	*sdh*	$<10^{-7}$	$<10^{-7}$
B. subtilis HS1A22	*sdh*	1.6×10^{-3}	1.4×10^{-3}
B. subtilis HS1A6	*fum*	2.7×10^{-3}	5.7×10^{-4}
B. subtilis HS1A8	*fum*	$<10^{-7}$	5.2×10^{-4}
B. subtilis HS1A21	*mdh*	$<10^{-7}$	$<10^{-7}$

[a] Symbols for each lesion are identified in Table 2.

[b] S/V is equal to the maximum thermostable counts observed divided by the total viable counts at $t_{0.5}$.

12% of the viable population. Several factors have been shown to affect the accuracy of the estimates of sporulation frequencies. The time of the maximum heat-stable count varies considerably with the mutant studied. The parent strain formed the maximum number of spores 25 hr after the end of logarithmic growth, and the number of spores remained constant for another 20 hr. In all of the mutants examined, the heat-stable count decreased after reaching a maximum. In one case, the maximum heat-stable count was observed 14 hr after logarithmic growth and decreased by two logs during the next 24 hr (*unpublished data*). All mutants lost viability after logarithmic growth was completed. Viable counts were taken immediately after logarithmic growth was completed, and sporulation frequencies reflect the fraction of the total population that formed spores rather than the fraction of the population that survived long enough to complete sporogenesis. Therefore, the results presented in Table 6 reflect a minimum estimate of the sporulation frequencies of the mutants.

Because media affected the synthesis of tricarboxylic acid cycle enzymes in some mutants in a manner different from effects observed with the parent strain, sporulation was also measured on different media. From the results of Table 6, it is also apparent that some mutants (particularly mutant HS1A12) sporulate better than the parent strain on heart infusion broth and sporulate better on this medium than on NSM. NSM is the better medium for sporulation of the parent strain.

DISCUSSION

The technique described for the isolation of tricarboxylic acid cycle mutants has proved to be a rapid and specific means for the isolation of mutants that do not exhibit recognizable pleiotrophic defects. All the tricarboxylic acid cycle mutants we have isolated produce normal zones of inhibition against *S. aureus* and *B. subtilis* strain H and normal proteinase activities against all substrates but elastin. Only two of the mutants isolated which produce halos on isolation medium and yellow colonies on purification medium have not been identified as tricarboxylic acid cycle mutants. One required hemoglobin for growth and has no recognizable lesion in the tricarboxylic acid cycle. It does not respond to heme precursors. The other mutant cannot convert ^{14}C-succinate to other tricarboxylic acid cycle intermediates and accumulates ^{14}C-succinate when incubated with ^{14}C-glutamate. Although apparently blocked in the conversion of succinate to fumarate, extracts of this mutant have normal levels of succinic dehydrogenase and succinate thiokinase. Using the technique described, we isolated previously undetected mu-

tants of *B. subtilis* lacking malate dehydrogenase and one with a modified citrate synthase activity. Citrate synthase mutants have not been reported in *B. subtilis*. The technique has been shown to be useful in the isolation of mutants that produce very low but detectable levels of tricarboxylic acid cycle enzymes.

Rutberg and Hock (11) reported that the genetic determinants for isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, fumarase, and succinic dehydrogenase are unlinked. Previous reports from this laboratory have indicated that these enzymes are not coordinately regulated in *B. subtilis* 168. Citrate synthase and aconitase are coordinately regulated and may belong to a single operon (2, 7). The isolation of mutants that are deficient in both of these enzymes supports the hypothesis that at least one gene regulates the synthesis of both proteins or that they share a protein in common.

The usefulness of mutants blocked in one reaction of the tricarboxylic acid cycle for the production of another enzyme is indicated by the results presented here and those of Rutberg and Hock (11). It is possible to greatly increase the specific activities of aconitase, isocitric dehydrogenase, and citrate synthase over those obtainable with the parent strain by using the appropriate medium and mutant.

Because two aconitase-negative mutants and one succinic dehydrogenase mutant isolated form spores in at least 1 to 10% of the viable cells, we can conclude that a functional tricarboxylic acid cycle is not essential to sporogenesis as previously proposed (13). It is also obvious that different methods of isolation may select tricarboxylic acid cycle mutants that differ in their abilities to express other postlogarithmic growth-phase events. We cannot explain why some tricarboxylic acid cycle mutants sporulate very poorly while others with the same lesion in the tricarboxylic acid cycle sporulate reasonably well. In each case in which sporulation was observed, we have shown that all of the surviving spores have retained the original marker and are not revertants. Freese and co-workers (5) proposed that poor sporulation of tricarboxylic acid cycle mutants results from a deficiency of ATP during sporogenesis. The aconitase-negative mutants isolated in this laboratory all show a drop in ATP levels after stationary phase. One that sporulates at frequencies above 1% maintains slightly higher ATP levels in stationary phase than those that sporulate poorly, but it has considerably lower levels of ATP than the wild type (A. A. Yousten and R. S. Hanson, unpublished data).

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