JOURNAL OF BACTERIOLOGY, Apr. 1968, p. 1431-1438
Vol. 95, No. 4

Printed in U.S.A.

Analysis of Sporulation Mutants

II. Mutants Blocked in the Citric Acid Cycle

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Received for publication 22 January 1968

Sporulation mutants that were unable to incorporate uracil during the developmental period recovered this capacity with the addition of ribose and in most cases with the addition of glutamate. Of the mutants that responded to both ribose and glumate, all but three also responded to citrate, and all but five responded to acetate. One of the exceptional strains was deficient in aconitase and another one in aconitase and isocitrate dehydrogenase; both required glutamate for growth. For the mutants which did not respond to glutamate, the products made from ^{14}C-glutamate were determined by thin-layer chromatography. Significant differences were found which enabled the identification of mutant blocks. The deficiency of the corresponding enzyme activity was verified. Several mutants were deficient in α-ketoglutarate dehydrogenase, and one lacked succinic dehydrogenase. These mutants could still grow on glucose as sole carbon source, but not on glutamate. The intact Krebs cycle is therefore not required for vegetative growth of aerobic *Bacillis subtilis*, but it is indispensable for sporulation.

Mutants unable to sporulate normally can be utilized to determine the biochemical pathways and control mechanisms of sporulation. Many sporulation mutants of *Bacillus subtilis* do not turn over ribonucleic acid (RNA) during the developmental period; this is shown by their inability to incorporate uracil into trichloroacetic acid-precipitable material (1, 2, 4, 11). Although all of these mutants incorporate uracil when ribose is added, some of them do not respond to other carbon sources. This lack of response can be employed to determine their biochemical lesions. In our previous paper (4), we showed that several mutants were nonresponsive to glutamate, indicating a biochemical block in the pathway between glutamate and ribose. One of these mutants lacked succinic dehydrogenase. In this paper, the mutant response of uracil incorporation to citrate and acetate is determined; enzyme deficiencies of several mutants blocked in the citric acid cycle are identified.

MATERIALS AND METHODS

Bacteria. B. subtilis sporulation mutants were derived from the transformable Marburg strain 60015 (methionine$^-$, indole$^-$), as described previously (4). Three mutants were single-colony isolates of strains kindly supplied: 60998 derived from ZD-2 Sp$_R$ of Spizizen, 60525 derived from the "aconitase$^-$" TT strain obtained from Szulmajster, and 61100 from the aconitase$^-$ TT strain obtained from Hanson. Strain 61100 was aconitase$^-$ and had normal isocitrate dehydrogenase activity, but this strain produced tough leatherlike colonies and clumps in liquid medium so that the measurement of cell concentrations by optical density (OD) was inaccurate. Strain 60525 produced normal growth curves and soft colonies, but it lacked both aconitase and isocitrate dehydrogenase; since the original strain apparently was deficient only in aconitase, our strain may be a double mutant. Our independently derived aconitase$^-$ mutant 60871 produced normal growth curves and soft colonies and produced isocitrate dehydrogenase.

Media and growth conditions. NSMP (nutrient sporulation medium) was prepared by autoclaving 8 g of Nutrient Broth (Difco) in 950 ml of distilled water. When the medium had cooled to below 60 C, 5 ml of autoclaved metal mixture, 0.5 ml of filter-sterilized $FeCl_3$ (2×10^{-3} M in 0.01 N HCl), and 50 ml of autoclaved potassium phosphate buffer (2 M, *p*H 6.5) were added in this sequence and were mixed after each addition. The metal mixture contained $CaCl_2$ (0.14 M), $MnCl_2$ (0.01 M), and $MgCl_2$ (0.2 M). All other media and growth conditions were the same as those used previously (4). In particular, NSM is NSMP without phosphate buffer.

To identify the growth stage of each culture we have used the notation T_n, where n is the time in hours elapsed from the moment the growth rate had started to decline.

^{14}C-uracil incorporation. ^{14}C-uracil was added to the culture to give a final concentration of 10^{-4} M and 0.15 μc/ml (uniformly labeled). Chloramphenicol was used

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at a final concentration of 50 μg/ml (stock solution of 20 mg/ml in acetone). Acetate and citrate were used as sodium salts (*p*H 6.5), at a final concentration of 2×10^{-3} M. The method employed for the incorporation of uracil into acid-precipitable material has been described previously (4).

Enzyme assays. To prepare bacterial extracts of cells grown in NSMP, the cells were harvested by centrifugation, washed with 0.05 M tris(hydroxmethyl)-aminomethane (Tris)-chloride buffer (*p*H 7.5), and lysed by 100 μg/ml of lysozyme. The cells were then treated with 2 μg/ml of deoxyribonuclease, and were finally centrifuged at 35,000 × *g* for 30 min at 0 C. Except for aconitase, the extracts were dialyzed against 0.02 M Tris-chloride buffer, *p*H 7.5. Aconitase was assayed immediately following lysis and centrifugation.

The activity of the citrate-condensing enzyme (citrate synthase = 4.1.3.7) was determined by the splitting of 5,5'-dithio-bis(2-nitrobenzoic acid) (DTN), which was caused by the coenzyme A (CoA) liberated from acetyl-CoA. The reaction mixture contained 2×10^{-2} M Tris-chloride buffer (*p*H 7.4), 10^{-2} M sodium oxalacetate, 5×10^{-4} M acetyl-CoA, and 10^{-3} M DTN. The reaction was followed at 412 mμ and was calibrated by CoA.

Reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase activity was assayed in a mixture containing Tris-chloride buffer, 2×10^{-2} M, and $NADH_2$, 10^{-3} M. All other enzymes were assayed by the methods given in Table 1. All the enzyme reactions were measured at 25 C in a volume of 1.0 ml.

Protein was determined by the biuret reaction (6).

The specific activities were calculated as micromoles or millimicromoles of substrate converted per minute and per milligram of protein. In all cases where NAD and $NADH_2$ reactions were involved, a correction was made for $NADH_2$ oxidase activity.

^{14}C-glutamate uptake. Log-phase cultures, grown in NSMP, were filtered through membrane filters (0.4-μ pore size, HA, Millipore Corp., Bedford, Mass.) and the bacteria were washed with an equal amount of minimal glucose (MG) medium at 37 C. The bacteria were resuspended at an OD at 600 mμ of 1.0 in prewarmed MG medium containing 50 μg/ml of chloramphenicol. After 15 min of shaking at 37 C, ^{14}C-L-glutamate (uniformly labeled) was added to give a final concentration of 2×10^{-4} M and 0.1 μc/ml. In 1-min intervals, samples of 0.5 ml were taken; the bacteria were collected on HA membrane filters and washed with 10 ml of prewarmed MG medium. The filters were then immediately dissolved in 10 ml of Bray's solution (3). The time needed for each sampling was less than 15 sec.

TABLE 1. *Assay methods for enzymes*

Name of enzyme	Classification no.[a]	Reference
Acetokinase	2.7.2.1	10
Aconitase	4.2.1.3	12
Isocitrate dehydrogenase	1.1.1.42	7
α-Ketoglutarate dehydrogenase		7
Phosphotransacetylase	2.3.1.8	13

[a] Refers to the recommendations (1964) of the International Union of Biochemistry.

Dissimilation of ^{14}C-glutamate. At $T_{2.7}$, ^{14}C-glutamate (uniformly labeled; specific activity, 167 mc/mmole) was added to 10 ml of the culture to give a final concentration of 2 μc/ml. After 5 min of incubation, the cells were centrifuged, resuspended in 4% of the original volume of water, and extracted by boiling for 30 min. The cells were removed by centrifugation, and the extracts were stored at −30 C. From 10 to 20 μliters of the extracts, corresponding to about 1 OD unit (600 mμ) of the bacterial culture, was applied to MN300 cellulose normal thin-layer plates (precoated, 250 μ, Analtech). The plates were chromatographed in the solvent systems described by Myers and Huang (9): ether-formic acid-water (7:2:1) and phenol (liquefied)-water-formic acid (75:25:1).

Autoradiographs were made by exposure to Kodak medical X-ray film (no screen) for 1 to 5 days.

For the detection of reference compounds, the plates were sprayed with bromocresol purple in 50% ethyl alcohol, *p*H 10 (5).

For densitometer tracing of the autoradiographs, a double-beam recording microdensitometer (model MK IIIB Joyce, Loebl) was used.

RESULTS

Uracil incorporation response to citrate and acetate. Most of the mutants which were unable to incorporate ^{14}C-uracil during the developmental period (at $T_{2.7}$) recovered this capacity with the addition of ribose, L-glutamate, or carbon compounds in the pathway(s) between ribose and α-ketoglutarate (4). Since they were apparently blocked in a pathway leading, via acetyl-CoA, to α-ketoglutarate, their response to citrate and acetate was also examined. (α-Ketoglutarate and isocitrate produced very little response for all strains tested because apparently they did not enter the cells at a sufficient rate.)

The time course of uracil incorporation (at $T_{2.7}$ in NSMP) is shown for two sporulation mutants in Fig. 1. Whereas strain 60875 (Fig. 1a) responded to all carbon sources, strain 60871 (Fig. 1b) exhibited no response to citrate or acetate. Mutant 60871 apparently was blocked between citrate and α-ketoglutarate. (It also showed a relatively low response to the other carbon sources, presumably owing to the same block.)

The uracil incorporation response to citrate and acetate was measured for all sporulation mutants 30 min after ^{14}C-uracil was added (at $T_{2.7}$ in NSMP). These results are tabulated in Table 2 and summarized in Fig. 2. Apart from the mutants which were already unable to respond to glutamate, only three mutants (60525, 60765, and 60871) did not respond to citrate and acetate.

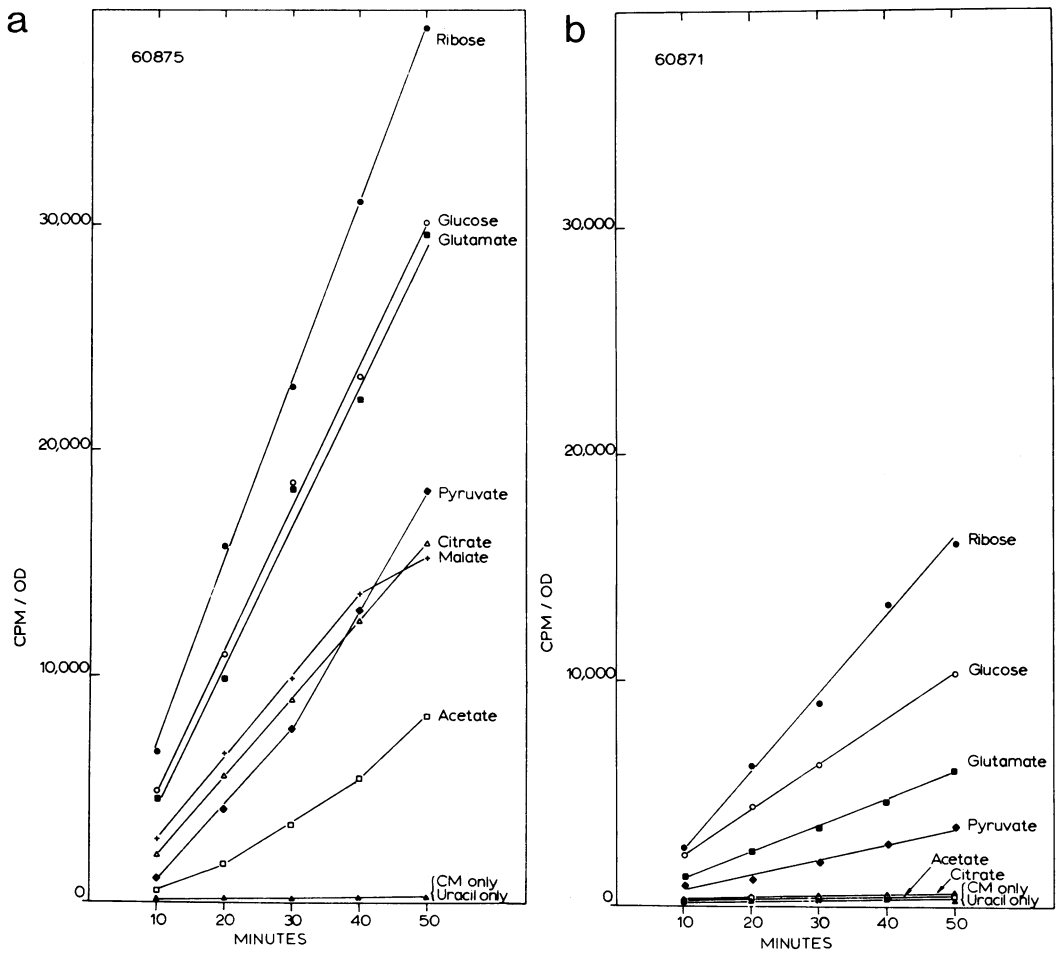

FIG. 1. *^{14}C-uracil incorporation into acid-precipitable material. At 2.7 hr after the growth rate had started to decline ($T_{2.7}$) in NSMP, the following compounds were added to the culture: ^{14}C-uracil only (▲); ^{14}C-uracil and chloramphenicol (CM only, ▲); ^{14}C-uracil, chloramphenicol, and 2 × 10^{-3} M ribose (●), glucose (○), glutamate (■), pyruvate (◆), citrate (△), malate (+), and acetate (□). Fig. 1a: strain 60875, Fig. 1b: strain 60871. The counts per minute of incorporated ^{14}C-uracil are standardized for the optical densities at 600 mμ of the cultures.*

Two other mutants (60722 and 60732) showed a low response to acetate but not to citrate.

Assay of enzymes between acetate and α-ketoglutarate. In the mutants which responded little or not at all to acetate, all enzymes were assayed (at $T_{2.7}$) that are needed for the conversion of acetate to α-ketoglutarate. It is apparent from Table 3 that only two mutants (60525 and 60871) lacked significant aconitase activity. In the mutant 60525, which was derived from an aconitase$^-$ strain obtained from Szulmajster (14), both aconitase and isocitrate dehydrogenase were absent.

Six independently isolated revertants of strain 60871, which grew on MG medium without glutamate, had normal aconitase activity and sporulated normally in NSMP. Similar to strain 60015, they also incorporated ^{14}C-uracil at $T_{2.7}$ without added carbon source. In addition, four strains, isolated as revertants for sporulation (by heating the culture and isolating brown colonies), could both sporulate on NSMP and grow on MG. Therefore, aconitase deficiency and inability to sporulate are controlled by the same mutation.

Characterization of mutants blocked between α-ketoglutarate and ribose. Several sporulation mutants examined in our previous paper (4) showed no uracil incorporation response to L-glutamate. To determine in which biochemical

TABLE 2. *Incorporation of ^{14}C-uracil into trichloroacetic acid-precipitable material*[a]

Strain	Chloramphenicol	Chloramphenicol + citrate	Chloramphenicol + acetate
60015	2,880	11,640	8,880
60646	2,840	13,080	8,120
60660	1,722	8,380	6,000
60709[b]	15,800	17,700	14,400
61102[b]	5,310	6,320	4,260
60818[b]	5,250	6,300	4,570
61105[b]	4,398	3,046	5,130
60815[b]	2,240	2,590	2,310
60813[b]	296	252	308
60998[b]	422		277
60705[b]	798	738	796
60790[b]	1,614	1,580	1,580
60763[b]	1,096	1,944	740
60726	3,344	3,140	3,000
60874	2,366	7,620	5,440
60871	390	332	388
60525	544	402	530
60765	464	412	478
60732	214	1,296	212
60722	312	4,080	312
60755	328	16,580	5,500
60793	300	16,000	6,100
60789	300	15,820	8,620
60829	340	15,820	8,460
60733	614	14,960	7,500
60806	1,820	14,760	6,900
60876	136	14,400	5,960
60645	590	14,080	7,500
60644	420	13,400	7,740
60805	374	13,020	7,540
60831	228	12,900	6,468
60804	234	12,800	6,450
60710	126	12,620	2,980
60651	216	11,760	6,760
60764	880	10,880	7,430
60703	252	10,400	5,700
60737	238	9,280	5,740
60725	462	8,320	3,560
60866	400	7,740	7,160
60693	298	7,700	4,060
60739	528	7,234	5,520
60875	246	7,100	1,900
60661	424	6,700	6,260
60759	232	6,890	5,740
60788	1,292	6,620	6,140
60735	488	6,600	3,580
60662	132	4,440	1,840

[a] Incorporation at $T_{2.7}$ in NSMP, *p*H 6.5. The values are corrected for the optical density (600 mμ) of the cultures (counts per minute per optical density unit). Strain 60015 is the sporulating control.

[b] Strains which showed no response to glutamate (4).

step they might be blocked, they were exposed at $T_{2.7}$ in NSMP to (uniformly labeled) ^{14}C-L-glutamate for 5 min. The cells were extracted, and the extract was applied to a cellulose thin-layer plate which was developed as described in Materials and Methods. Figure 3 shows an autoradiograph after 3-day exposure, whereas Fig. 4 shows the densitometer tracing of a similar picture after 2-day exposure. Long exposure had the advantage of revealing trace amounts of radioactivity, although it obscured the separation between citrate and glutamate.

The mutants displayed in Fig. 3 can be divided into three groups. The first group contained 60015, which is the standard strain, and 60722 and 60732, which had the same pattern as the standard strain but exhibited a larger amount of succinate and phosphoenolpyruvate. These strains apparently are partially blocked or differently controlled in some enzymes. In the second group was strain 60763, which accumulated succinate and did not show any of the compounds between succinate and glucose or ribose. This result agrees with the finding in our previous paper (4) that 60763 is blocked in succinic dehydrogenase. Nevertheless, glutamate could still be metabolized to citrate in this mutant (Fig. 4), but citrate could not be efficiently converted to oxalacetate. The third group contained strains 60705, 60790, 60813, 60818, 60998, and 61105, which all showed large amounts of glutamate and citrate and small amounts of α-ketoglutarate. None of the other compounds was present to any significant extent. One of the mutants (60813) showed especially small amounts of citrate and α-ketoglutarate. This strain also demonstrated a low rate of glutamate uptake measured at $T_{2.7}$ in NSMP (Fig. 5), in contrast to strain 60015. Strain 60813 possibly is deficient in a permease for L-glutamate; this could be ascertained only by a nonmetabolizable analogue of L-glutamate. Several other strains (60660, 60703, 60739, and 60788), for which uracil incorporation responds to acetate, exhibited the same pattern of ^{14}C-glutamate dissimilation as did 60015.

Assay of α-ketoglutarate dehydrogenase. The specific activity of α-ketoglutarate dehydrogenase increased during growth in NSMP, as was assayed by the reduction of NAD (Fig. 6). After growth had ceased, the activity seemingly declined, but simultaneously a nonmembrane-bound $NADH_2$ oxidase activity increased in amount. The latter probably oxidized the small amount of $NADH_2$, produced by the α-ketoglutarate dehydrogenase, so effectively that hardly any activity

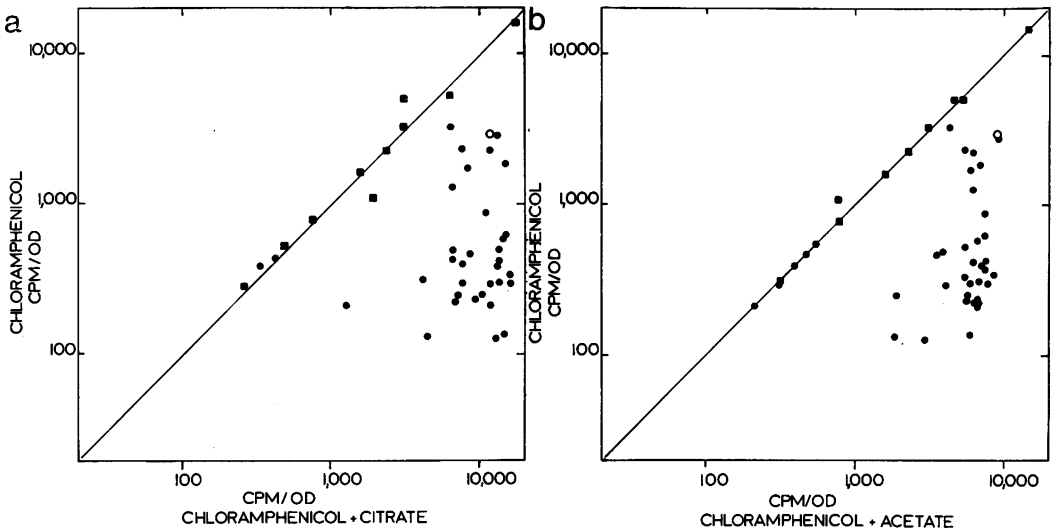

FIG. 2. *Rates of ^{14}C-uracil incorporation into acid-precipitable material for the standard strain 60015 (○), sporulation mutants which do respond to L-glutamate (●), and those which do not respond to L-glutamate (■). The cultures were grown to $T_{2.7}$ in NSMP. The pH was adjusted to 6.5 with 1 N HCl, and the cultures were incubated with ^{14}C-uracil (10^{-4} M, 0.15 μc/ml), chloramphenicol (50 μg/ml), and citrate or acetate (2×10^{-3} M) for 30 min at 37 C. Fig. 2a: incorporation with citrate. Fig. 2b: incorporation with acetate. The counts per minute values are standardized for the optical density (at 600 mμ) of the cultures.*

TABLE 3. *Specific activity of several enzymes in sporulation mutants*[a]

Strain	Isocitrate dehydrogenase	Aconitase	Citrate synthase (condensing enzyme)	Phosphotransacetylase	Acetokinase
60015	279	178	4.0	64	129
60525	<2	<1	3.1	107	84
60662	230	198	1.7	88	129
60732	47	103	2.8	93	77
60763	187	144	2.9	61	54
60818	111	70	5.5	88	53
60871	89	<1	4.4	82	52
60875	268	178	3.1	75	292

[a] All specific activities are calculated as millimicromoles of substrate converted per minute and per milligram of protein at 25 C. Bacteria were harvested at $T_{2.5}$. The activities for aconitase and isocitrate dehydrogenase are corrected for NADP H_2 oxidase activity.

of α-ketoglutarate dehydrogenase was observed after T_2.

To determine whether some of the above mutants lacked α-ketoglutarate dehydrogenase, their specific activity was measured at three times—T_{-1}, T_0, and T_1. Table 4 shows that all strains had a significantly reduced α-ketoglutarate dehydrogenase activity, which explains all properties of these mutants—inability to grow on L-glutamate as sole carbon source, inability to convert ^{14}C-glutamate into other carbon compounds following α-ketoglutarate in the Krebs cycle, and absence of a uracil incorporation response to glutamate. In addition to its low ^{14}C-glutamate uptake, strain 60813 also showed a low specific activity for α-ketoglutarate dehydrogenase.

To establish the correlation between the different mutant properties, seven sporulating revertants of strain 60705 were isolated by their brown color production on NSM plates. They exhibited the normal specific activity for α-ketoglutarate dehydrogenase and they could grow on glutamate as sole carbon source.

Growth on different carbon sources. The ability of different compounds to serve as sole carbon sources was determined by inoculating 60015 at 10^6 cells/ml in tubes containing N (+ Met + Trp) and 10^{-2} M of the carbon compounds (neutralized) to be tested. The following compounds produced growth: citrate, fumarate, glucose, glutamate, glycerol, malate, oxalacetate, pyruvate, and ribose. In contrast, neither acetate nor succinate gave growth, even after 3 days of incubation.

DISCUSSION

We have shown that several sporulation mutants lack the activity of one of four different

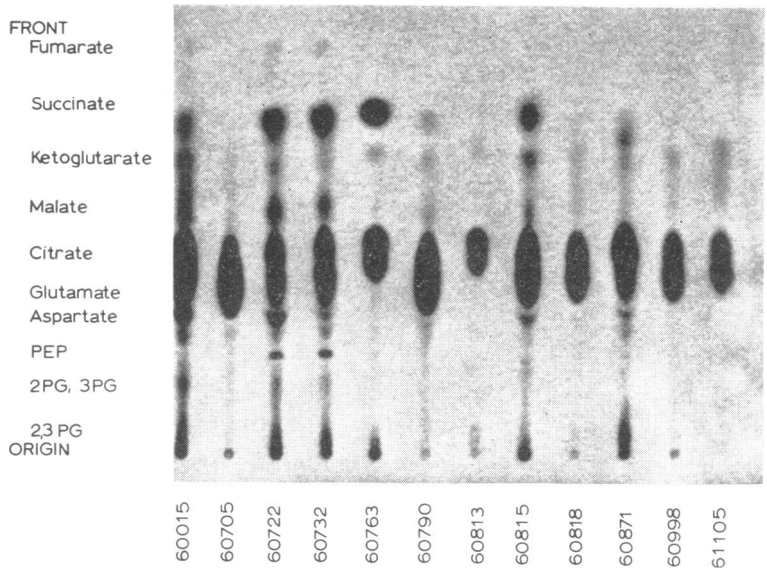

FIG. 3. *Autoradiography of ^{14}C-L-glutamate dissimilation products of the standard strain 60015 and several sporulation mutants. ^{14}C-glutamate was added at $T_{2.7}$ for 5 min. Thin-layer chromatography on MN 300 cellulose in the solvent system ether-formic acid-water (7:2:1). Kodak no screen X-ray film was exposed for 3 days. PEP = phosphoenolpyruvate; 2PG = 2-phosphoglycerate; 3PG = 3-phosphoglycerate; 2,3PG = 2,3-phosphodiglycerate.*

Krebs cycle enzymes—aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, or succinic dehydrogenase. Szulmajster and Hanson (14) found that a glutamate-requiring mutant which lacked aconitase activity was unable to sporulate normally. Our independently isolated aconitase$^-$ mutant and the double mutant, aconitase$^-$-isocitrate dehydrogenase$^-$, also required glutamate for growth. In contrast, all mutants deficient in α-ketoglutarate dehydrogenase or succinic dehydrogenase can still grow on glucose minimal medium. Apparently, vegetative growth of the aerobic *B. subtilis* does not depend critically on the intact Krebs cycle, whereas sporulation does. In this cycle, the enzymes between succinyl-CoA and oxalacetate are all reversible so that the metabolic intermediates can be formed from glucose, even when α-ketoglutarate dehydrogenase is missing. However, α-ketoglutarate dehydrogenase itself is sufficiently irreversible so that aconitase mutants require the addition of glutamate for growth. When ^{14}C-glutamate is added to a mutant deficient in α-ketoglutarate dehydrogenase or succinic dehydrogenase, ^{14}C-citrate is produced because the enzymes between citrate and glutamate are also readily reversible. The conversion of oxalacetate + acetyl-CoA to citrate, however, is irreversible in *B. subtilis* under our growth conditions (i.e., citrate lyase is missing) because none of the phosphorylated intermediates of the Embden-Meyerhof pathway were found to be radioactive, and also because the above mutants could not grow on glutamate as sole carbon source.

The inability of α-ketoglutarate dehydrogenase-deficient mutants to grow on glutamate as sole carbon source indicates that the glyoxalate pathway is not operating in *B. subtilis*. The same conclusion can also be drawn when *B. subtilis* is unable to grow on acetate as sole carbon source, although ^{14}C-acetate is taken up, and acetate must be processed via the citric acid cycle as it restores uracil incorporation in many sporulation mutants. Growth on acetate is possible only in the presence of a functional glyoxalate bypass by which new carbon compounds can be derived from acetate because in the citric acid cycle itself acetate is quantitatively converted to CO_2 (8).

The biochemical reactions known to operate during the developmental period are shown in Fig. 7. This figure indicates also the enzymes at which different mutants are blocked.

The ability to incorporate uracil into RNA during the developmental period could be restored by ribose (or glucose) for all our sporulation mutants. Evidently, the intact Krebs cycle is not absolutely required for RNA synthesis as long as energy (adenosine triphosphate) can be produced elsewhere (in the Embden-Meyerhof pathway or the hexose monophosphate shunt). Nevertheless, all mutants blocked in the Krebs cycle exhibited, even in the presence of ribose, a lower rate of uracil incorporation than did other

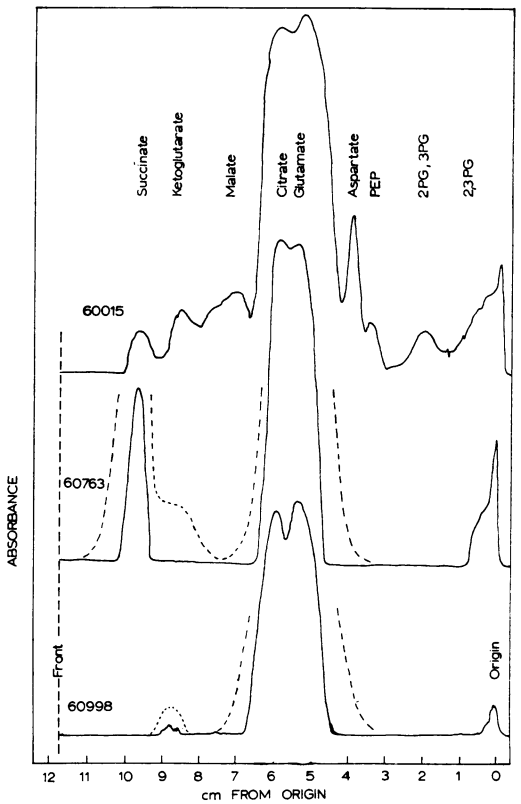

FIG. 4. *Density tracing of autoradiography of ^{14}C-glutamate dissimilation products. Chromatography was the same as in Fig. 3, but film exposure was 2 days (solid line) and 4 days (dashed line). Strain 60015 = standard strain, 60763 = succinic dehydrogenase$^-$, and 60998 = α-ketoglutarate dehydrogenase$^-$.*

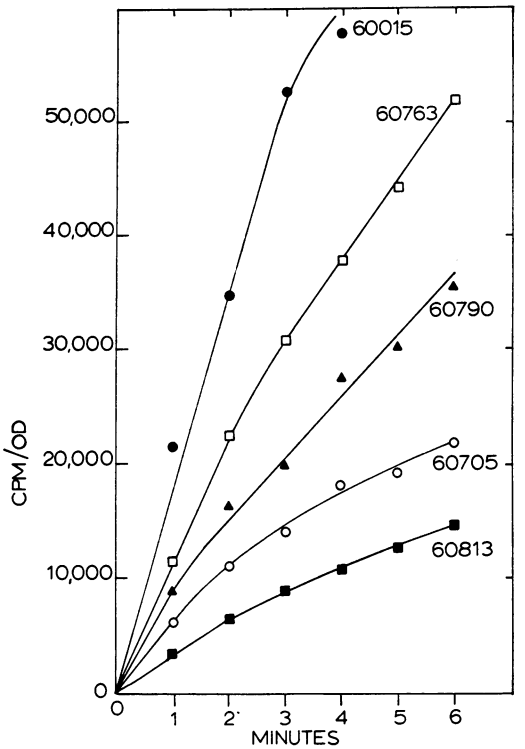

FIG. 5. *^{14}C-glutamate uptake of standard strain 60015 (●), and of spore mutants 60763 (□), 60790 (▲), 60705 (○), and 60813 (■). The counts per minute values of incorporation are standardized for the optical density (600 mμ) of the cultures.*

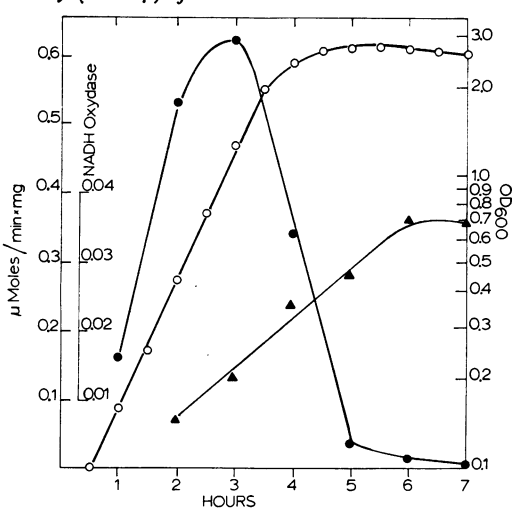

FIG. 6. *α-Ketoglutarate dehydrogenase activity during growth of standard strain 60015 in nutrient sporulation medium. The specific activity is calculated as micromoles of $NADH_2$ produced per minute and per milligram of protein at 25 C. Left ordinate: specific activity of enzymes, α-ketoglutarate dehydrogenase (●), $NADH_2$ oxidase (▲); right ordinate: optical density (600 mμ) of the culture (○).*

sporulation mutants. The amount of adenosine triphosphate produced from ribose or glucose obviously is smaller when the Krebs cycle is blocked than when it is completely operative because some carbon intermediates accumulate that cannot be completely oxidized. During the developmental period, however, the complete Krebs cycle is indispensable, apparently because sufficient amounts of adenosine triphosphate cannot be produced otherwise.

For each of the Krebs cycle mutants, the capacity to incorporate uracil during the developmental period is restored by all carbon compounds which can enter the pathway between the blocked enzyme and ribose or glucose. Thus, the earliest blocked mutant (aconitase$^-$) responds to glutamate, succinate, fumarate, malate, oxalacetate, pyruvate, and similar compounds but not to citrate or acetate. Apparently, the natural carbon precursor, used during the developmental period, enters the Krebs cycle via acetyl-CoA and citrate. The production of this

TABLE 4. *α-Ketoglutarate dehydrogenase activity in sporulation mutants*[a]

Strain	T_{-1}	T_0	T_1
60015	644	368	66
60705	<10	<9	<10
60790	58	30	45
60813	20	20	20
60818	<8	<10	<10
60998	<7	<8	<9
61105	<4	<5	<7

[a] All specific activities are calculated as millimicromoles of $NADH_2$ formed per minute and per milligram of protein at 25 C and corrected for $NADH_2$ oxidase activity. Bacteria were harvested at times T_{-1}, T_0, and T_1.

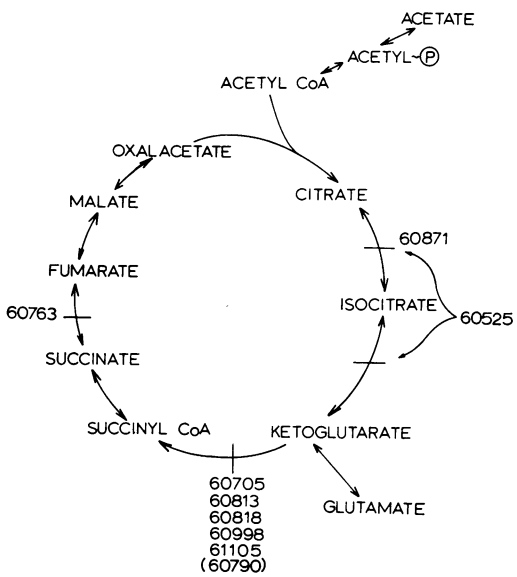

FIG. 7. *Scheme of substrate conversion during sporulation of Bacillus subtilis. Enzymes blocked in the citric acid cycle are indicated by the numbers of the mutants.*

precursor or its conversion to acetyl-CoA seems to be blocked in those sporulation mutants which respond to citrate or acetate. These mutants are also unable or hardly able to produce an antibiotic which is made early in sporulation (4).

ACKNOWLEDGMENT

We are indebted to Uta Fortnagel for her excellent assistance in this study.

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