GLUCOSE CATABOLISM BY *BACILLUS POPILLIAE* AND *BACILLUS LENTIMORBUS*¹

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ABSTRACT

PEPPER, ROLLIN E. (Michigan State University, East Lansing), AND RALPH N. COSTILOW. Glucose catabolism by *Bacillus popilliae* and *Bacillus lentimorbus*. J. Bacteriol. 87:303-310. 1964.—Resting cells of *Bacillus popilliae* and *B. lentimorbus* catabolize glucose with the production of CO₂, lactic acid, acetic acid, glycerol, ethanol, and trace amounts of acetoin and acetaldehyde. The first three products are the major ones, and their ratios may be varied by controlling the availability of oxygen. Practically no lactic acid is produced when oxygen is not limiting, whereas it may comprise up to 80% of the total acid when oxygen is greatly limited. However, no glucose is catabolized by resting cells in the absence of molecular oxygen. Isotope and inhibitor studies and assays for key enzymes of the established metabolic routes all indicate that these organisms utilize both the Embden-Meyerhof and hexosemonophosphate pathways for glucose dissimilation. With a concentrated resting-cell suspension, the extent of participation of the latter route was estimated to be as high as 40% in an atmosphere of pure oxygen, and as low as 2% in air. Acetate was oxidized by only one of the cultures of *B. popilliae* tested, which is apparently a mutant. Cells of this strain from stationary phase cultures oxidized acetate at pH 7.0 or higher, but not at pH 6.0; however, they oxidized succinate, fumarate, and malate more rapidly at pH 6.0 than at 7.0. The oxidation of tricarboxylic acid cycle intermediates, the presence of condensing enzyme in extracts of cells capable of oxidizing acetate, and the complete inhibition of acetate oxidation by arsenite and partial inhibition by malonate all indicate that terminal oxidation of acetate by this strain of *B. popilliae* is via the tricarboxylic acid cycle.

Bacillus popilliae and *B. lentimorbus* both cause the "milky disease" of the Japanese beetle (*Popillia japonica*) larvae (Dutky, 1940). The parasitic cycle, as it appears in the larvae,

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was extensively discussed by Dutky (1940), Beard (1945), and Angus and Heimpel (1960). These organisms grow poorly in vitro, as compared with most other members of this genus. Dutky (1947) reported that *B. popilliae* utilizes glucose and fructose; Steinkraus (1957) found that other carbohydrates could be utilized. Both of these workers alluded to extensive acid production by their references to pH drop in growth media and to the need for high buffering capacity.

B. popilliae and *B. lentimorbus* are obviously different from several members of this genus in that they require complex media for growth and do not sporulate to any extent in vitro. It was thought that a study of their basic catabolic processes might reveal some basic differences and aid in devising media and conditions conducive to sporulation. The present investigation was designed to determine the products of glucose oxidation by these organisms and to elucidate the dissimilatory pathways involved.

MATERIALS AND METHODS

B. popilliae NRRL B-2309-P and *B. lentimorbus* NRRL B-2522, obtained from the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill., were used in these studies. The culture of *B. popilliae* used is a mutant, and differs from the parent culture in that it oxidizes acetate. Another culture of the same strain maintained in lyophil, and obtained from the same source, does not possess this characteristic, nor does another culture of this strain maintained on agar slants in this laboratory. Therefore, this culture is now designated as NRRL B-2309-P-A. This culture was tested in the Northern Utilization Research and Development Division laboratories and found to be still capable of producing typical "milky disease" of Japanese beetle larvae.

Cultures were maintained in a medium, recom-

mended by Harlow H. Hall (*personal communication*), which contained 1.5% yeast extract, 0.2% glucose, and 0.6% K_2HPO_4 . This was dispensed in 250-ml amounts in 500-ml Erlenmeyer flasks. Cultures were incubated at 30 to 32 C on a rotary shaker, and transfers to fresh medium were made at intervals of 24 to 72 hr.

Cell suspensions were prepared by separating cells from the growth medium by centrifugation, washing twice, and resuspending in distilled water. Samples (1 ml) of cell suspensions were dried at 106 C for 24 hr for determination of dry weights.

Cell extracts were prepared by breaking cells with no. 100 glass beads (Minnesota Mining and Manufacturing Co., St. Paul, Minn.) in a high-speed Servall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.). Approximately 12 g (wet weight) of cells were suspended with 40 to 45 g of glass beads in 50 ml of distilled water. The mixture was chilled for 30 min in an ice bath; the blender cup was maintained in the ice bath throughout the breaking period, which was completed in 10 to 15 min. The extract was centrifuged at $3,000 \times g$ for 15 min and dialyzed 15 to 18 hr against distilled water at 4 C. The protein content was determined by the method of Lowry et al. (1951).

Oxygen consumption and CO_2 evolution were measured manometrically with a Warburg respirometer by the procedures outlined by Umbreit, Burris, and Stauffer (1957). Flask contents were examined for initial and residual substrate, as well as for metabolic products.

$C^{14}O_2$ produced from glucose-1- C^{14} and -6- C^{14} by resting cells was collected in 20% KOH contained in a small vial placed in the center well of a Warburg vessel. The method described by Goldman (1961) was used to collect $C^{14}O_2$ resulting from the oxidation of acetic acid-1- C^{14} by a growing culture. Flasks (500 ml) containing 25 ml of the yeast extract-glucose medium and 0.5 μ c of isotope were plugged with rubber stoppers. Each stopper was outfitted with a bent glass rod to which a tube (1.2 by 3.5 mm) was secured with a rubber band. The tube, suspended above the medium, contained 0.5 ml of 40% KOH to trap the CO_2 . Inoculated flasks were shaken at 200 rev/min at 30 to 32 C, and individual flasks were removed periodically for analysis. The radioactivity of CO_2 and of samples of the residue clarified by centrifugation was measured by placing 1-ml samples into the gel

scintillation system of Gordon and Wolfe (1960), and by counting disintegrations with a Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Co., La Grange, Ill.).

Glucose was assayed by the colorimetric determination of reducing sugars described by Neish (1952). Total acids were determined by direct titration. Specific types of acids were identified by chromatographic separation (Wiseman and Irvin, 1957). Also, lactic acid was determined colorimetrically, and volatile acid by distillation, as outlined by Neish (1952). Glycerol was oxidized to formaldehyde by periodate (Neish, 1952), and a method described by Nash (1953) was used to quantitate the formaldehyde from which glycerol was calculated. Ethanol was determined by the microdiffusion procedure, acetaldehyde by bisulfite binding, and acetoin by titration with alkaline iodine, as described by Neish (1952). Pyruvate was estimated by the colorimetric test detailed by Umbreit et al. (1957).

Glucose-6-phosphate (G-6-P) dehydrogenase was assayed by following the reduction of nicotinamide adenine dinucleotide phosphate (NADP; Kornberg and Horecker, 1955). Activities of other hexosemonophosphate (HMP) pathway enzymes were demonstrated by measuring triose phosphate production from ribose-5-phosphate (R-5-P) by trapping them with hydrazine and converting them to their phenylhydrazones (Sibley and Lehninger, 1949). The method of Heath et al. (1958) was used to assay for 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, and that of Kovachevich and Wood (1955) was used for phosphoketolase. Aldolase was estimated colorimetrically (Sibley and Lehninger, 1949); triose phosphate isomerase activity was indicated by a modification of the same procedure in which the trioses were not reacted with hydrazine until after the incubation period. Phosphofructokinase was assayed by another modification of the aldolase assay in which fructose-6-phosphate (F-6-P) and adenosine triphosphate (ATP) were substituted for fructose-1,6-diphosphate (F-1,6-P). Acetokinase was determined by the appearance of acetyl phosphate from acetate and ATP (Rose, 1955). Phosphotransacetylase was determined by the disappearance of acetyl phosphate in the presence of coenzyme A (CoA) and arsenate (Stadtman, 1952). The condensing enzyme was determined by the disappearance of acetyl phosphate when arsenate was omitted, and when CoA and oxal-

acetate were supplied (Ochoa, Stern, and Schneider, 1951). Acetyl phosphate was measured by the method of Lipman and Tuttle (1945).

RESULTS

Products of glucose catabolism. Products of glucose oxidation by *B. popilliae* were examined for the presence of organic acids by column chromatography. Bands corresponding to lactic and acetic acids descended the column, whereas none corresponding to butyric, propionic, formic, or succinic acids appeared. These results correspond closely to those obtained by direct tests for lactic acid and for volatile acids; the acids recovered accounted for essentially all of the titratable acidity. CO₂ was the only other major product of glucose catabolism, but there were measurable amounts of glycerol and ethanol and traces of acetoin and acetaldehyde. A carbon balance of glucose oxidation by resting cells is given in Table 1. Over 96% of the carbon of the

TABLE 1. Carbon balance of glucose oxidation by resting cells of *Bacillus popilliae**

Material†	Amt	Percentage of utilized glucose	C ₃ utilized (μmoles per 100 μmoles)	Carbon
	μg			μmoles
Lactic acid	3,960	55.5	55.4	166.0
Acetic acid	1,190	16.7	25.0	50.0
CO ₂	1,670	23.4	47.9	47.9
Ethanol	311	4.4	8.5	16.9
Glycerol	68	0.5	2.8	8.4
Acetoin	Trace	—	—	—
Acetaldehyde . .	Trace	—	—	—
Total amount . .	7,199		139.6	289.2‡

* Oxidations were run in Warburg vessels in an air atmosphere. The main compartment contained 53.2 mg of cells suspended in 0.05 M phosphate buffer (pH 7.0); 0.5 ml of 0.08 M glucose was tipped from a side arm after thermal equilibrium, and 0.2 ml of 4 N H₂SO₄ was tipped from a side arm at the end of the incubation period; 0.2 ml of 20% KOH was added to the center well to determine O₂ uptake. The total liquid volume was 3 ml. CO₂ was determined manometrically, and other products, as outlined in Materials and Methods. Endogenous controls were run, and all values were corrected accordingly.

† Total amount of glucose added was 7,200 μg. Of this, all but 70 μg were oxidized.

‡ Carbon recovery = $\frac{289.2}{300} = 96.4\%$.

FIG. 1. Effect of oxygen levels upon the amounts of lactic and acetic acids produced by resting cells of *Bacillus popilliae*. Oxidations were run in Warburg cups at 30 C. Reaction mixtures contained 48.8 mg (dry weight) of cells harvested from a 24-hr culture, 0.5 ml of 0.3 M phosphate buffer (pH 7.0), and 0.5 ml of 0.067 M glucose; 0.2 ml of 20% KOH was present in the center well. The liquid volume was 3 ml. Oxygen levels were adjusted by mixing with helium on a per cent by volume basis.

glucose oxidized was accounted for among five products, indicating that all major products were identified.

Upon repeating the carbon balance with varying cell concentrations, different proportions of lactic and acetic acids were observed, although the total acidity remained constant. It was found that the ratio of these acids could be varied over a wide range by changing the oxygen level in the atmosphere. As the percentage of oxygen increased, acetic acid production increased and lactic acid production decreased (Fig. 1). However, glucose was not dissimilated under anaerobic conditions, and lactic acid never accounted for more than 82% of the total acid. All of the acid produced by a growing culture incubated on a rotary shaker was volatile.

Pyruvate was anaerobically fermented to approximately equimolar amounts of acetic and lactic acids and of CO₂. No hydrogen was evolved.

Pathways of glucose oxidation. Preliminary evidence of the catabolic pathways used by *B. popilliae* was obtained through the use of inhibitors. Iodoacetate (0.01 M) inhibited glucose

oxidation 100%, 0.01 M fluoride inhibited approximately 50%, and 0.1 M fluoride inhibited completely. Because iodoacetate inhibits glyceraldehyde-3-phosphate dehydrogenase, and because low concentrations of fluoride inhibit enolase, the data indicate that the lower part of the Embden-Meyerhof sequence (EMP) is required for glucose catabolism. It appears unlikely that either the phosphoketolase or Entner-Doudoroff systems are present, because they should be insensitive to iodoacetate.

Assays for key enzymes of the known pathways of glucose catabolism were then performed on cell-free extracts of *B. popilliae*. No KDPG aldolase or phosphoketolase activity was found. This is in agreement with the inhibitor studies, because these are key enzymes of the Entner-Doudoroff and phosphoketolase pathways, respectively.

G-6-P dehydrogenase, a key enzyme of the HMP system, was present in dialyzed cell extracts. The enzyme was dependent upon NADP and Mg^{++} (Fig. 2); nicotinamide adenine dinucleotide (NAD) would not function as a coenzyme. 6-Phosphogluconate decarboxylation was indicated by the production of $C^{14}O_2$ prefer-

FIG. 2. G-6-P dehydrogenase activity in an extract of *Bacillus popilliae* cells. Cuvettes contained dialyzed extract, and 1 ml of 0.04 M glycyl glycine buffer (pH 7.4). To the reactions indicated were added 2 μ moles of G-6-P, 0.9 μ mole of NADP, and 20 μ moles of $MgCl_2$. The total volume was adjusted to 3 ml.

TABLE 2. Triose production by an extract of *Bacillus popilliae* cells with R-5-P as substrate*

Extract	Δ Optical density at 540 m μ †
Blank	0
Protein, 3 mg	0.09
Protein, 9 mg	0.30

* Reaction mixtures contained 25 μ moles of R-5-P, 1.0 ml of 0.1 M tris(hydroxymethyl)amino-methane buffer (pH 8.6), 0.25 ml of 0.56 M hydrazine (pH 8.0), extract, and water to 3.0 ml. An incubation period of 30 min was allowed, and the reaction was stopped by introducing 2.0 ml of 12% trichloroacetic acid. The blank contained all reagents, including 9 mg of protein, and was treated the same as the tests, except that the R-5-P was added at the end of the incubation period after addition of trichloroacetic acid. Triose phosphates were determined by the method of Sibley and Lehninger (1949).

† Increase in optical density reflects production of triose phosphates from R-5-P.

entially from glucose-1- C^{14} , as compared with glucose-6- C^{14} . The other enzymes of the HMP system were not assayed individually, but were undoubtedly present, because triose phosphates were formed on incubation of R-5-P with a cell extract (Table 2). Dihydroxyacetone phosphate production from R-5-P was also demonstrated by the assay method of De la Haba and Racker (1955), with only R-5-P as substrate.

The presence of enzymes of the upper half of the EMP was established by measuring the appearance of triose phosphates from various substrates by the colorimetric procedure of Sibley and Lehninger (1949, Table 3). With F-6-P and ATP present, the appearance of triose phosphates was indicative of the presence of both phosphofructokinase and aldolase. The increased optical density (OD), observed on addition of hydrazine after incubation of the reaction mixture containing F-1,6-P as substrate, indicates the presence of triose phosphate isomerase, because dihydroxyacetone phosphate results in a more highly colored phenylhydrazone than does the glyceraldehyde-3-phosphate. Glucokinase reactions, and those enzymatic systems required to convert glyceraldehyde-3-phosphate to pyruvate, would be required for both the EMP and HMP systems; thus, these were not assayed directly. There was no glucose oxidase or glucose dehydrogenase detectable in the cell-free extracts.

Based on the isotopic method of Wang et al. (1958), the percentage of glucose catabolized by a concentrated resting-cell suspension via phosphogluconate decarboxylation could be varied greatly by controlling the availability of oxygen. With 60 mg (dry weight) of cells per 3 ml in an air atmosphere, the calculated participation of the HMP system was 2%; whereas, with 48 mg (dry weight) of cells per 3 ml in an atmosphere of pure oxygen, it was about 40%.

Oxidation of acetate and tricarboxylic acid cycle intermediates. Initial attempts to oxidize acetate with resting cells of *B. popilliae* failed. Later it was found that cells from stationary-phase cultures of one strain would oxidize this substrate at neutral and alkaline pH levels. Acetate inhibited endogenous respiration at pH 6.0 to 6.1, but was oxidized at reasonably rapid rates at pH levels of 7.0 to 8.0. Conversely, succinate, fumarate, and malate were all oxidized more rapidly at pH 6.1 than at pH 7.4 to 7.6 (Table 4).

Cells harvested from a culture in the log phase of growth (9 hr of incubation) had little acetate-oxidizing ability; the Q_{O_2} of cells of this age was 0.27, as compared with a Q_{O_2} of 1.4 for cells from stationary-phase cultures. Similar results were obtained with growing cultures by following the appearance of $C^{14}O_2$ from acetate- $1-C^{14}$ dur-

TABLE 3. Aldolase, triose phosphate isomerase, and phosphofructokinase activities in extracts of *Bacillus popilliae* cells

Substrate	Cell extract	Δ Optical density at 540 $m\mu$ *
	ml	
None.....	0.1	0.00
	0.5	0.00
F-1,6-P.....	0.1 (A)†	0.38
	0.1 (B)	0.66
F-6-P‡.....	0.5	0.41

* Change in optical density at 540 $m\mu$ reflects an increase in triose phosphates as determined by the method of Sibley and Lehninger (1949). A 30-min incubation period was used.

† Hydrazine was present in reaction mixture A during incubation, but it was not added to B until after incubation for 30 min. Thus, triose phosphate isomerase was active in the B mixture.

‡ Hydrazine was not added to the reaction mixture until after incubation. Thus, all three enzymes would be active.

TABLE 4. Oxidation of acetate and tricarboxylic acid intermediates by resting cells of *Bacillus popilliae*

Substrate*	Concn	Q_{O_2} † at pH:			
		6.0 to 6.1	7.0	7.4 to 7.6	8.0
Endogenous	M	0.45	0.46	0.48	0.66
Acetate	0.01	—0.10	0.69	0.46	0.51
	0.04	—0.23	0.74	1.40	1.99
Succinate	0.04	0.11	—	0.05	—
Fumarate	0.04	0.06	—	0.04	—
Malate	0.04	0.90	—	0.04	—

* All activities on substrates were corrected for endogenous rates, which were determined manometrically. Warburg vessels contained 33 to 35 mg (dry weight) of cells from a 24-hr culture, and 0.5 ml of 0.2 M phosphate buffer at desired pH; 0.2 ml of 20% KOH was in the center well; substrates adjusted to yield the indicated concentrations were tipped in from a side arm after thermal equilibrium. The final liquid volume was 3 ml. The reactions were run in an O_2 atmosphere at 30 C, for time periods from 90 to 180 min.

† Q_{O_2} = μ liters of O_2 per hr per mg (dry weight) of cells.

ing incubation. No significant amount of labeled CO_2 was liberated until after 24 hr of incubation.

Acetokinase, phosphotransacetylase, and condensing enzyme were demonstrated in extracts of *B. popilliae* cells. However, the condensing enzyme was not found in extracts of cells incapable of oxidizing acetate (Table 5).

These data, plus the facts that the respiratory quotient for acetate oxidation was 1, and that the oxidation was completely inhibited by arsenite and partially by malonate, indicated that the culture of *B. popilliae* used in this work oxidized acetate via the tricarboxylic acid cycle. A functional glyoxylate bypass was apparently not present, because arsenite completely inhibited acetate oxidation.

Studies with B. lentimorbus. Manometric studies with resting cells of *B. lentimorbus* yielded results which were almost identical to those obtained with *B. popilliae*, except for acetate oxidation. Most of the carbon from glucose appeared as CO_2 and lactic and acetic acids; the proportions of each depended on oxygen availability. Glucose was not fermented anaerobically. Iodoacetate and fluoride inhibited glucose oxidation by *B. lentimorbus* to a degree similar

TABLE 5. *Acetokinase, phosphotransacetylase, and condensing enzyme activities in extracts of Bacillus popilliae cells*

Enzyme(s) assayed	Reaction mixture*	Extract†	Δ Optical density at 540 mμ‡
Acetokinase	Complete	None	0.00
		A, 3.0 mg of protein	0.34
		A, 6.0 mg of protein	0.70
		B, none	0.00
		B, 2.3 mg of protein	0.17
		B, 6.9 mg of protein	0.42
Phosphotransacetylase and condensing enzyme	Complete	None	0.00
	Minus oxalacetate	A, 8.8 mg of protein	0.00
	Minus CoA	A, 8.8 mg of protein	0.00
	Complete	A, 4.4 mg of protein	-0.15
	Complete	A, 8.8 mg of protein	-0.23
	Complete	B, none	0.00
	Complete	B, 2.3 mg of protein	0.00
	Complete	B, 6.9 mg of protein	0.00
Phosphotransacetylase	Complete	None	0.00
	Minus CoA	B, 2.3 mg of protein	0.00
	Complete	B, 6.9 mg of protein	-0.11

* See Materials and Methods for methods used.

† Extract A was of cells of the acetate-oxidizing culture, and extract B was of cells of parent culture which did not oxidize acetate.

‡ Change in optical density at 540 mμ reflects a change in the level of acetyl phosphate present, as determined by the method of Lipmann and Tuttle (1945).

to that of *B. popilliae*. Isotopic data indicated the presence of both EMP and HMP systems; the extent of participation of the latter was controlled by adjusting oxygen levels. Pyruvate was anaerobically dissimilated with the evolution of CO₂, but not of hydrogen. Acetate was not oxidized by resting cells.

DISCUSSION

There is nothing obviously unique about the pathways of glucose catabolism employed by *B. popilliae* and *B. lentimorbus*. All of the evidence obtained from enzyme assays and isotope and inhibition studies indicates that both the EMP and HMP systems are active. This is typical of a great number of aerobic and facultatively anaerobic microorganisms (Cheldelin, 1961). The relative importance of the two systems in growing cultures was not determined, but the data with resting cells indicate that the participation of the HMP may be controlled by varying the availability of oxygen. This may well be due to the necessity of molecular oxygen for the oxidation of reduced NADP which is formed by the G-6-P dehydrogenase. It is likely that reduced NAD can

be reoxidized in the formation of the lactate, ethanol, or glycerol which were found to be present. The great increase in lactate production when oxygen is limited to resting cells indicates that this is true.

The failure of *B. popilliae* and *B. lentimorbus* cells to ferment glucose in the absence of oxygen is of considerable interest. These organisms were reported to be facultative anaerobes and to grow best in highly poised reducing media (Dutky, 1940). However, we failed to obtain significant growth in the complete absence of molecular oxygen, and found aeration of broth cultures to be very stimulatory to growth. The Marburg strain of *B. subtilis* will not grow anaerobically, but cells grown in complex media will ferment glucose to lactic acid (Gary and Bard, 1952). *B. popilliae* and *B. lentimorbus* have the enzymatic system for lactate production, as evident from the high yields of lactate when oxygen is limited and from their ability to dismutate pyruvate in the absence of oxygen. The reason for the failure of resting cells of these organisms to ferment glucose is not evident.

The failure of a number of strains of *B. popilliae*

and *B. lentimorbus* to oxidize acetate, and the failure of the strain of the former which did oxidize this substrate to do so at acid pH, may be reasons for the lack of success in sporulating these organisms in vitro. Nakata and Halvorson (1960) and Hanson, Srinivason, and Halvorson (1962) demonstrated that acetate oxidation is initiated in cultures of *B. cereus* just prior to sporulation. The appearance of acetate oxidation in cultures of *B. cereus* described by Hanson et al. (1962) was similar to that observed in this work, except that *B. cereus* will oxidize acetate at low pH levels. Therefore, it may be necessary to select cultures of these two species that oxidize acetate, and to grow them in media with the pH maintained at neutrality, or above, to obtain spores in vitro.

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