Physiology of Sporeforming Bacteria Associated with Insects: Metabolism of *Bacillus popilliae* Grown in Third-Instar *Popillia japonica* Newman Larvae

GRANT ST. JULIAN, LEE A. BULLA, JR.,* AND RICHARD S. HANSON

Northern Regional Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Peoria, Illinois 61604 and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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The timing and relative participation of concurrent pathways of carbohydrate metabolism as well as the extent of terminal respiratory activity were determined by radiorespirometry with $^{14}$C substrates and by enzyme assays for vegetative and sporulating cells of the bacterium *Bacillus popilliae* cultured in whole, intact *Popillia japonica* (Japanese beetle) larvae. During vegetative proliferation, the pentose phosphate pathway predominates in the bacterial cells with minor involvement of the Embden-Meyerhof-Parnas pathway. As the cells proceed through sporulation, pentose phosphate and Embden-Meyerhof-Parnas activity remains constant. No tricarboxylic cycle activity is evident during growth and sporulation of *B. popilliae*. The results demonstrate (i) predominantly aerobic metabolism for carbohydrate assimilation within in vivo sporulating cells, (ii) a major contrast to the metabolism of other aerobic sporeforming bacteria that exhibit derepression of tricarboxylic acid cycle enzymatic activity at the onset of sporulation, and (iii) no causal necessity of the cycle to *B. popilliae* sporogeny.

Spores of the bacterium *Bacillus popilliae*, when ingested by *Popillia japonica* (Japanese beetle) larvae, germinate and outgrow in the digestive tract. The resulting vegetative cells invade the hemolymph, where they proliferate and ultimately form billions of spores before causing death of the insect host. The term "milky disease" refers to the milky appearance of the hemolymph that becomes heavily laden with spores subsequent to larval death. The spores permit the bacterial pathogen to survive long periods in the soil and are the means of further disease transmission. Such attributes render the organism an effective microbial insecticide.

The factors that prompt sporulation of *B. popilliae* within and without an insect host are not yet clearly understood. Unlike related aerobic sporeforming bacteria, *B. popilliae* does not sporulate appreciably in artificial culture (17). Furthermore, it does not display the metabolic changes that occur in certain *Bacillus* species during transition from vegetative growth to sporulation (11). In order to establish the biochemical events that accompany sporulation of *B. popilliae*, fundamental knowledge must be gained of the metabolism of both host and pathogen as well as the regulatory mechanisms that control bacterial morphogenesis during the infectious process. With this goal in mind, we investigated the activity of central metabolic pathways associated with biosynthesis and energy production in vegetative and sporulating *B. popilliae* cells harvested directly from experimentally diseased, third-instar Japanese beetle larvae. We present in this paper data that elucidate operation of simultaneous pathways of glucose catabolism and terminal respiration in the differentiating bacterial cells, and the temporal relationship of such metabolic activities with spore development in *B. popilliae*. (This paper was presented, in part, at the 25th annual meeting of the American Institute of Biological Sciences, Arizona State University, Tempe, Arizona, 16 to 21 June 1974.)

MATERIALS AND METHODS

Infection of larvae. Third-instar larvae of the Japanese beetle were collected in Ohio. Their care and handling have been described (19, 20). About 1.5 x $10^8$ *B. popilliae* spores were injected into the hemolymph of individual larvae to induce milky disease according to the procedure of St. Julian and Hall (19). After injection, the larvae were incubated at 28 C in loam soil previously seeded with rye grass. Healthy (uninfected) larvae were used as controls.

Collection of hemolymph and bacteria. Milky

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* Present address: U.S. Grain Marketing Research Center, ARS, USDA, Manhattan, Kan. 66502.
disease develops in four phases (18, 21). There is an initial incubation phase (phase I) of about 2 days in which relatively few bacterial cells are apparent; from day 3 to day 5 (phase II), vegetative cells rapidly proliferate and by day 5 a few prespore forms occur as well as an occasional spore; phase III marks an intermediate change (days 5 to 10) from predominantly vegetative growth to prespore and spore development; thereafter, a sporulation phase (phase IV) that terminates by day 14 to day 21 involves massive sporulation and ensuing larval death. Pooled hemolymph samples (30 ml) were collected as described by Shotwell et al. (22) from uninfected larvae controls and from infected third-instar larvae at phases II and III of disease. The hemolymph samples were combined with 30 ml of cold 0.02 M phosphate buffer (pH 7.4). B. popilliae cells were separated from the infected hemolymph by centrifugation at 4°C. Cell purity was monitored by direct microscopy observation and by screening for contaminating colonies from hemolymph inoculated on dilution plates containing solid medium. After being washed three times in cold 0.02 M phosphate buffer (pH 7.4), the cells were suspended at a concentration of 3 mg/ml (dry weight) in the phosphate buffer and stored at −20°C for subsequent studies.

**Enzyme assays.** A French pressure cell was used to prepare cell extracts. About 90% of the cells were ruptured by this method. Protein was determined on the cell extracts by the method of Lowry et al. (12). Unless otherwise indicated, enzyme activities were assayed spectrophotometrically at 340 nm by standard procedures (7). A Beckman DU-2 spectrophotometer was used for all spectrophotometric determinations. Enzyme activities are expressed as enzyme units per milligram of protein in the cell-free extracts. One enzyme unit is defined as the quantity of enzyme that converts 1 μmol of substrate per min at 25°C.

Phosphoenolpyruvate-glucone-phosphate phosphotransferase was assayed by the procedure of Ghosh and Ghosh (9). Glucokinase (adenosine triphosphate d-glucose 6-phosphotransferase, EC 2.7.1.2) was measured by observing nicotinamide adenine dinucleotide (NAD) reduction in the presence of excess glucose 6-phosphate dehydrogenase. Glucose 6-phosphate dehydrogenase (NAD;glucose-6-phosphate:nicotinamide adenine dinucleotide phosphate [NADP] oxidoreductase, EC 1.1.1.49), and 6-phosphogluconate dehydrogenase (6-phospho-δ-glucuronate: NADP oxidoreductase [decarboxylating], EC 1.1.1.44) were determined by NADP reduction.

Transketolase (p-sedoheptulose-7-phosphate: d-glyceraldehyde-3-phosphate, EC 2.2.1.1) activity was determined by following reduced NAD (NADH) oxidation in a triose isomerase-α-glyceraldehyde dehydrogenase coupled reaction that forms glyceraldehyde-3-phosphate from ribose-5-phosphate.

The assay for 6-phosphogluconate dehydrogenase (6-phospho-δ-glucuronate hydro-lyase, EC 4.2.1.12) involved coupling its activity with endogenous 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase in the presence of excess lactic dehydrogenase and following the reduction of pyruvate by NADH. The substrate was 6-phosphogluconate. By using KDPG as substrate, KDPG aldolase (6-phospho-2-keto-3-deoxy-δ-gluconate d-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.14) also was measured by observing the reduction of pyruvate by NADH. KDPG was kindly supplied by W. A. Wood, Dept. of Biochemistry, Michigan State University, East Lansing, Mich.

Assay for glucose dehydrogenase (β-d-glucose:NAD oxidoreductase, EC 1.1.1.47) activity was based on measurement of NAD reduction in the presence of excess glucose.

Phosphofructokinase (ATP:δ-fructose-6-phosphate-1-phosphotransferase, EC 2.7.1.11) was assayed by coupling with fructose-1,6-diphosphate aldolase, triosephosphate isomerase, and glyceraldehyde dehydrogenase and following NADH oxidation. Fructose-1,6-diphosphate aldolase (fructose-1,6-diphosphate d-glyceraldehyde-3-phosphate-lyase, EC 4.1.2.13) activity was determined by following NADH oxidation during formation of triose phosphates from fructose-1,6-diphosphate in the presence of excess glyceraldehyde dehydrogenase and triosephosphate isomerase.

The following tricarboxylic acid cycle enzymes were assayed as reported previously (6): citrate synthase (citrate oxaloacetate-lyase [coenzyme A acetylating], EC 4.1.3.7); aconitase (citrate [isocitrate] hydratase, EC 4.2.1.3); isocitrate dehydrogenase (NADP) (threo-δ-isocitrate: NADP oxidoreductase, EC 1.1.1.42); succinate dehydrogenase (succinate: fumarase (L-malate hydro-lyase, 4.2.1.2); and malate dehydrogenase (L-malate: NAD oxidoreductase, EC 1.1.1.37).

**Radiorespirometric methods.** Radiorespirometric experiments, each done in triplicate (5), were performed on the B. popilliae cells removed from hemolymph, washed three times by centrifugation, and suspended in 0.02 M phosphate buffer (pH 7.4). Radiorespirometry also was done with bacterial cells in hemolymph and hemolymph alone. Specifically labeled d-[14C]glucose was obtained from New England Nuclear Corp., Boston, Mass. Specifically labeled pyruvate, acetate, succinate, glutamate, and α-ketoglutarate were purchased from International Chemical and Nuclear Corp., Waltham, Mass. Respirometer flasks containing 30 mg of cells were incubated at 28°C in a modified Gilson differential respirometer; as a control, hemolymph minus cells was incubated in the same way. Specifically labeled substrate (0.25 μCi) was added from a side arm; each flask contained 0.1 mg of radioactive substrate. Final volume of material per flask was 30 ml. Flasks were shaken while air was passed through at a rate of 60 ml/min. To decrease endogenous metabolism, cultures were incubated for 60 min before addition of substrate. Respiratory 14CO2 was trapped in 10 ml of a mixture of absolute ethyl alcohol and monoethanolamine (2:1, vol/vol); the ethyl alcohol-ethanolamine trapping solution was removed and replenished at 0.5-h intervals.

At the end of each experiment, the culture flasks were chilled quickly, and cells were separated by centrifugation at 4°C. Cells were homogenized in NCS
solubilizer (Amersham/Searle Corporation, Des Plaines, Ill.) and incubated at 37°C for 12 h; incorporated label was then counted in a scintillation mixture containing (vol/vol) toluene-2, 5-diphenyloxazole-1, 4-bis-2,5-phenyloxazolyl-benzene/Triton X100: ethyl alcohol (8:4:3). Samples of the phosphate buffer and hemolymph also were counted in this scintillation mixture. Counting efficiency for each sample was determined by appropriate internal standards.

Total 14CO2 recoveries from [4-14C]glucose were calculated from total recoveries of [3-14C]glucose and [3,4-14C]glucose. The C4 recovery data were calculated as C4 = 2(C3, 4) – (C3).

RESULTS

Metabolism of milky disease phase II and phase III cells. Radiorespirometric patterns of glucose and pyruvate oxidation are presented in Fig. 1 for phase II (vegetative) and phase II (sporulating) cells removed from hemolymph. Total isotope recoveries in respired CO2, cells, and medium from catabolism of labeled substrates are shown in Table 1.

Both vegetative and sporulating cells displayed the same pattern of carbon dioxide evolution (Fig. 1A and B). The differential rates of CO2 evolution from specifically labeled glucose were C-1 > C-2 > C-4 > C-3 > C-6. Preferential oxidation to CO2 of C-1 over C-6 and of C-4 over C-3 is evidence for operation of the pentose phosphate (PP) pathway. Extensive

![Diagram](image)

**Fig. 1.** Radiorespirometric patterns for oxidation of glucose and pyruvate by milky disease phase II (vegetative, A) and phase III (sporulating, B) cells of Bacillus popilliae. C-1, C-2, C-3, C-4, and C-6 designate specifically labeled carbon atoms. One-tenth of a milligram of specifically labeled substrate (0.25 μCi) was added to each reaction flask. Final volume per flask was 30 ml.
conversion of C-2 to CO$_2$ is indicative of extreme cycling of carbons via pentoses. In such a scheme, oxidative decarboxylation is catalyzed by 6-phosphogluconate dehydrogenase in which 6-phosphogluconate is converted to ribulose-5-phosphate. The resulting pentose phosphate is either further metabolized to glyceraldehyde-3-phosphate or is recycled to form hexose phosphate which, in turn, again may be catabolized. The carbonyl carbon of glyceraldehyde-3-phosphate produced by reactions catalyzed by transaldolase and transketolase would correspond to the C-4 of the parent glucose molecule; the first hydroxyl carbon of the resulting fructose-6-phosphate would be the original C-2 of the glucose. CO$_2$ results from conversion of glyceraldehyde-3-phosphate to pyruvate and its oxidation to acetyl coenzyme A. Evolution of C-2 as CO$_2$ would result from pentose cycling reactions in which 6-phosphogluconate is decarboxylated to ribulose-5-phosphate. The conversion of C-4 and C-3 to CO$_2$ indicates participation of the Embden-Meyerhof-Parnas (EMP) pathway. In this pathway, glucose is converted to two molecules of pyruvate whose carboxyl groups correspond with carbons three and four of glucose. Subsequent oxidation produces C-4 and C-3 as CO$_2$ before the other carbons of pyruvate are oxidized.

The radiorespirometric patterns for use of glucose were similar for both vegetative (disease phase II) and sporulating (disease phase III) cells. Relative participation of concurrent pathways differed insignificantly. Use of glucose via the PP pathway varied from 75% in phase II cells to 80% in phase III cells; EMP participation varied from 25 to 20%.

Elaboration of acetate-oxidizing systems is a metabolic event that correlates with the transition from vegetative growth to sporulation of certain *Bacillus* species. Organic acids that accumulate during growth are oxidized via the tricarboxylic acid cycle. Apparently this cycle provides the energy necessary for subsequent biosynthesis and sporulation. To determine whether a tricarboxylic acid operates during sporulation of *B. popilliae*, oxidation of specifically labeled pyruvate, acetate, succinate, glutamate, and α-ketoglutarate was tested with phase II and phase III cells. As can be seen in Table 1, no $^4$CO$_2$ was evolved from acetate, succinate, glutamate, and α-ketoglutarate; oxidation of pyruvate to CO$_2$ involved only C-1, not C-2 and C-3.

Specific activities of enzymes of the PP, EMP, Entner-Doudoroff, and tricarboxylic acid cycle pathways are listed in Table 2. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, enzymes that catalyze the NAD-requiring PP reactions exhibited higher specific activities than did the EMP enzymes phosphofructokinase and fructose-1,6-

### Table 1. Isotope recoveries for catabolism of labeled glucose and oxidation of labeled pyruvate, acetate, succinate, glutamate, and α-ketoglutarate by milky disease phase II (vegetative) and phase III (sporulating) cells of *B. popilliae*

| Substrate | Phase II | Phase III |
|-----------|----------|-----------|
|           | CO$_2$   | Cells     | Medium* | CO$_2$ | Cells | Medium* |
| [1C,$^14$C]glucose | 32       | 48       | 20      |        | 23     | 56       | 21 |
| [2C,$^14$C]glucose | 19       | 62       | 19      |        | 12     | 65       | 23 |
| [3C,$^14$C]glucose | 7        | 67       | 26      |        | 3      | 78       | 23 |
| [4C,$^14$C]glucose | 6        | 63       | 31      |        | 2      | 74       | 21 |
| [6C,$^14$C]glucose | 1        | 71       | 28      |        | 0.5    | 73       | 28 |
| [1C,$^13$C]pyruvate | 7        | 63       | 29      |        | 5      | 60       | 33 |
| [2C,$^13$C]pyruvate | 0        | 72       | 22      |        | 0      | 74       | 32 |
| [3C,$^13$C]pyruvate | 0        | 67       | 31      |        | 0      | 59       | 44 |
| [1C,$^12$C]acetate | 0        | 55       | 44      |        | 0      | 58       | 44 |
| [2C,$^12$C]acetate | 0        | 61       | 32      |        | 0      | 67       | 33 |
| [3C,$^12$C]acetate | 0        | 73       | 32      |        | 0      | 81       | 26 |
| [1C,$^12$C]glutamate | 0        | 64       | 33      |        | 0      | 39       | 62 |
| [2C,$^12$C]glutamate | 0        | 58       | 52      |        | 0      | 38       | 65 |
| [3C,$^12$C]glutamate | 0        | 50       | 56      |        | 0      | 41       | 50 |
| [5C,$^12$C]glutamate | 0        | 62       | 38      |        | 0      | 47       | 51 |
| [1C,$^12$C]α-ketoglutarate | 0        | 43       | 54      |        | 0      | 32       | 63 |

* Average of three separate experiments; incubation was for 3 h.

* Medium is phosphate buffer as described in Materials and Methods.

* Numbers designate specifically labeled carbon atoms.
Phosphoenolpyruvate:glucose phosphotransferase activity in Phase II and Phase III cells of B. popilliae 

| Enzyme                                | Phase II | Phase III |
|---------------------------------------|----------|-----------|
| Phosphoenolpyruvate:glucose phosphotransferase | <0.10    | <0.10     |
| Glucokinase                           | 37       | 30        |
| Glucose-6-phosphate dehydrogenase     | 22       | 18        |
| 6-Phosphogluconate dehydrogenase      | 43       | 47        |
| Transketolase                         | 32       | 35        |
| 6-Phosphogluconate dehydrogenase      | <0.05    | <0.05     |
| KDPG aldolase                         | <0.05    | <0.05     |
| Phosphofructokinase                   | 21       | 17        |
| Fructose-1,6-diphosphate aldolase     | 18       | 13        |
| Glucose dehydrogenase                 | <0.10    | <0.10     |
| Citrate synthase                      | <0.10    | 0.03      |
| Aconitase                             | 0.50     | 0.80      |
| Isocitric dehydrogenase               | 0.02     | 0.02      |
| Succinic dehydrogenase                | 0.17     | 0.28      |
| Fumarase                              | 40       | 40        |
| Malic dehydrogenase                   | 0.90     | 1.80      |

*Cells removed from Japanese beetle larvae hemolymph.

Values are expressed as 10^{-4} enzyme units per milligram of protein. To calculate net specific activities of enzyme reactions involving either NADH or NADPH oxidation, NADH oxidase and NADPH oxidase activities were subtracted from the respective total activity values. Due to interference by NADH oxidase and presumably low activity of α-ketoglutarate dehydrogenase, the latter enzyme is not reported.

diphosphate aldolase; 6-phosphogluconate dehydrase and KDPG aldolase, enzymes critical to operation of the Entner-Doudoroff pathway, exhibited essentially no activity. Significantly, specific activities of the PP and EMP pathways were comparable in phase II and phase III cells. Phosphoenolpyruvate:glucose phosphotransferase and glucose dehydrogenase, both lacking in the sporeformer Bacillus larvae (16), were not evident in B. popilliae.

The lack of a fully operational tricarboxylic acid cycle in B. popilliae is exemplified by the absence of citrate synthase and the extremely low levels of aconitase, isocitric dehydrogenase, and succinic dehydrogenase. Similar results were obtained with in vitro cultured cells reported by Yousten et al. (24).

Metabolism of diseased hemolymph. No $^{14}$CO$_2$ was evolved from radioactive glucose added to hemolymph alone, previously collected from milky diseased Japanese beetle larvae (phases I through IV). Furthermore, there was no oxidative decarboxylation of C-1 of pyruvate in the hemolymph. Healthy (control) hemolymph exhibited no oxidative activity with any of the substrates tested.

DISCUSSION

The relatively high specific activities of key enzymes of the PP and EMP pathways in B. popilliae corroborate radioisoporic metabolic signatures that reflect concomitant participation of these two pathways. Assays of six tricarboxylic acid cycle enzymes support earlier conclusions (4, 5) that B. popilliae (NRRL B-2309) does not possess a fully operational cycle. Other investigators (13) have demonstrated that variant oligosporogenous strains of B. popilliae oxidize acetate in vitro. Our data indicate that the bacterium does not require activation of a complete tricarboxylic acid cyclic to sporulate in vivo. To what extent the pathogen may depend upon the host's metabolism is not known; intact healthy and diseased Japanese beetle larvae possess an active tricarboxylic acid cycle (3).

The mechanism by which glucose is oxidized in vegetative cells of several aerobic sporeforming bacteria involves the EMP and PP pathways (1, 2). The EMP pathway is the primary route for glucose assimilation whereas the PP pathway, by supplying NADH, aids formation of biosynthetic intermediates rather than functioning as a major respiratory pathway. In contrast, B. popilliae grown within the insect host assimilates glucose predominantly via the PP pathway with minor involvement of the EMP pathway. Instead of relying on terminal respiratory activity for energy production and biosynthesis during sporulation, in vivo cells of B. popilliae apparently utilize the PP pathway and associated pentose cycling reactions. Interestingly, Pepper and Costilow (15) and Bulla et al. (5) observed primary participation of the EMP pathway for in vitro grown cells. We have no explanation for such a dramatic difference between in vivo and in vitro pathway participation. Perhaps enzymes of the EMP pathway could be repressed by a compound(s) in the insect hemolymph and that derepression of certain EMP pathway enzymes occurs once the organism is removed from its host and cultured artificially.

Earlier, we postulated (5) that biosynthetic
events during the growth of *B. popilliae* in vitro hampers activation of a tricarboxylic acid cycle and that, in turn, spore formation is excluded. Attempts to derepress the cycle enzymes in vitro were unsuccessful (4). Although activation of a tricarboxylic acid cycle has been implicated as a requisite for sporulation (8, 10), Yousten and Hanson (23) and Nickerson et al. (14) demonstrated that the tricarboxylic acid cycle is not an absolute requirement for sporulation of *B. subtilis* and *B. thuringiensis*, respectively. As was true for these two microorganisms, our data establish that the tricarboxylic acid cycle is inoperative during in vivo sporulation of *B. popilliae* and obviously the cycle is not required. The parameters that initiate in vivo sporulation of *B. popilliae* have not been defined. We believe that sporulation within the insect is host dependent.

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