Oxidation of Acetate by Various Strains of Bacillus popilliae

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A number of strains of Bacillus popilliae were examined for their ability to oxidize acetate. Some of these would not sporulate in vitro, and some were oligosporogenous. The ability to oxidize acetate varied widely among the strains tested. A culture derived from spores of the parent strain produced in vivo and one of the asporogenous strains derived from it failed to produce significant levels of $^{14}$CO$_2$ from $[^6]$Cacetate. Oligosporogenous strains derived from the same parent culture all produced $^{14}$CO$_2$ from both $[1-^{14}]$C and $[2-^{14}]$C acetate but at relatively low rates. The highest rates of acetate oxidation were observed with three strains which did not produce spores in vitro. When cultured under appropriate conditions, one of these strains displayed a secondary growth response concomitant with a decrease in the titratable acidity and an increase in the pH of the medium. The data indicate that B. popilliae has a complete citric acid cycle but that the activity of the cycle is strongly repressed in wild-type strains under the usual conditions used for in vitro cultivation.

Acetate oxidation after vegetative growth is characteristic of a number of aerobic spore-forming bacilli and is important in their sporogenic process (1, 7). Mutants deficient in acetate oxidation of a number of species are asporogenic (1, 6). However, some tricarboxylic acid cycle mutants of Bacillus subtilis sporulate "nearly as well as the prototroph" (5). Bacillus popilliae, an insect pathogen, metabolizes glucose during growth with the accumulation of CO$_2$ and acetic acid (12). However, most strains previously tested failed to sporulate in vitro and also failed to oxidize the acetate accumulated. One strain was observed by Rhodes et al. (13) to be oligosporogenous when cultured on a solid medium containing critical amounts of sodium acetate, but it is not known whether the acetate was oxidized under the conditions where spores were formed. Another strain has been studied which did oxidize acetate to a variable extent, but in no instance was sporulation formation observed (12). Recently, a strain sporulating at a significant frequency was described (16), but no information is available with respect to its ability to oxidize acetate.

The objective of this study was to determine whether there is a correlation between the ability of B. popilliae strains to sporulate and oxidize acetate. We examined a number of strains for acetate oxidation and found considerable variation among them. However, all strains which sporulate to a detectable extent in vitro also oxidize acetate. The medium and conditions of growth are critical with respect to acetate oxidation.

MATERIALS AND METHODS

Cultures and cultural methods. The strains of B. popilliae used in this study are listed in Table 1 along with their original sources and differentiating characteristics. As indicated, the parent culture (NRRL B-2309) and a number of the variants were obtained from the Northern Utilization Research and Development Division (NRRL), Agricultural Research Service, Peoria, Ill.

The mutants isolated in our laboratory (MS-54, MS-69, MS-85) were derived from a culture of NRRL B-2309 which was sent to us in the spore stage by the NRRL laboratory. This strain had been sporulated by inoculating Japanese beetle larvae, and the culture was received in dried hemolymph on a glass slide. The vegetative culture was initiated by suspending a small amount of the dried material in sterile water, heat shocking at 60°C for 15 min, and then inoculating flasks containing 1.5% Trypticase, 0.5% yeast extract, 0.2% glucose, and 0.6% K$_2$HPO$_4$ (TYG) broth (9) with decimal dilutions of the suspension. The culture obtained from the highest dilution was characteristic of B. popilliae and was used in our studies. The oligosporogenous mutants were isolated after treating...
Table 1. Strains of Bacillus popilliae used in various experiments

| Strain          | Relevant comments                                                                 |
|-----------------|------------------------------------------------------------------------------------|
| No spores       |                                                                                    |
| formed in vitro |                                                                                    |
| NRRL B-2309 (8) | Parent strain of all cultures used. Our culture was derived from spores produced in vivo. |
| NRRL B-2309S (13) | Original variant which formed spores on solid media, but we have never observed any spore formation. Our culture has been maintained on slants for over 3 years. |
| NRRL B-2309N (16) | Variant culture isolated from B-2309M. Our culture maintained on slants. Cells are elongated as compared to parent culture. |
| NRRL B-2309MC   | Culture derived from B-2309M after cultivation in broth for several months in our laboratory. Growth rate is higher than the parent culture. |
| NRRL B-2309PA (12) | Culture maintained as a broth culture in our laboratory for many years. It was found to oxidize acetate several years ago. |
| Spores formed  |                                                                                    |
| at low frequencies in vitro |                                                                                    |
| NRRL B-2309M (16) | Cultures used were produced from heat-shocked spores produced in colonies on plates. Culture was derived from B-2309S. |
| MS-54, MS-69, MS-85 | Mutants from culture of B-2309 isolated as described in the text. Cultures used were derived from heat-shocked spores produced in colonies on plates or in broth cultures. |

*All cultures were derived from B-2309 which was isolated originally from commercial spore dust (8).*

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a log-phase culture in TYG broth medium with N-methyl-N'-nitro-N-nitrosoguanidine (0.2 mg/ml). After 15, 30, 60, and 90 min of exposure, 10 ml of this culture was removed. The cells were centrifuged and washed with fresh medium one time and then inoculated into flasks of 1% Mueller Hinton, 1% yeast extract, 0.3% phosphate, 0.05% glucose (MYPG) broth. This medium is the same as the sporulation medium of Sharpe et al. (16) except glucose is used instead of trehalose. After incubating these flasks on a shaker for 2 to 3 weeks, 1-ml sub-samples were removed, placed in 2 ml of melted soft agar (0.75%), tempered at 60°C, and held at this temperature for 20 min. These were then poured on the surface of MYPG plus 0.1% pyruvate agar in petri dishes. The colonies which developed were examined for spores, and those having the largest numbers were selected. Inoculation of MYPG-agar plates by the procedures described by Sharpe et al. (16) demonstrated that these cultures produced sporangia containing refractile spores and paraspores at the low frequencies (2 to 6%) which we observe with 2309M under these conditions.

Cultures which were maintained in liquid medium were transferred every 2 or 3 days in TYG broth and incubated on a shaker. Those maintained on slants were on JB medium (14). Slant cultures were incubated for 3 to 4 days, stored at 5°C, and transferred at 2-week intervals. All cultures used were typical of *B. popilliae* since (i) there were characteristic birefringence of broth cultures and the absence of a ring on the flask, (ii) they were nonmotile rods, (iii) vegetative cells were catalase-negative, and (iv) they failed to grow on nutrient agar.

**Growth experiments.** Cultures in TYG medium were incubated at 30°C on a rotary shaker. Samples were removed at intervals for analysis. Optical density at 620 nm was measured in a Gilford model 2000 spectrophotometer with the uninoculated medium as a blank. Glucose was estimated by measuring total reducing sugar (11) and total volatile acids determined by titration of the acid after steam distillation of acidified samples.

**Oxidation of various substrates.** Cells were harvested, washed, and suspended in sufficient 0.05 M potassium phosphate (pH 7.4) to result in an optical density at 620 nm of 10. Dry weights were determined by drying 1-ml samples of these suspensions at 110°C to constant weight and correcting for the phosphate present. The suspensions routinely contained between 10 and 15 mg (dry weight) per ml. Oxygen-uptake studies were conducted in a Warburg respirometer at 30°C. The flasks contained 1 ml of the cell suspension, 1 ml of 0.05 M potassium phosphate (pH 7.4), and substrate(s) at the concentrations indicated below. The center well of the Warburg flasks contained 0.2 ml of 20% KOH. In experiments with labeled acetate as substrate, the 14CO2 was trapped on a fluted 2 by 2 cm piece of Whatman no. 1 filter paper saturated with 10% KOH (3). Sulfuric acid (0.3 ml of 2 M) was tipped into the reaction mixture from a side arm at the end of the incubation period to release all of the 14CO2, and the flasks were incubated 1 hr longer. The 14CO2 was then counted by dropping the filter paper into a vial containing the scintillation solution of Bray (2). Appropriate controls to correct for [14C]acetate distillation were included in each experiment.

**Incorporation of 14C from [U-14C]acetate.** Reac-
Production and utilization of acetate during growth. The production and utilization of acid by two strains of *B. popilliae* 2309PA and 2309M growing in a highly buffered medium are illustrated in Fig. 1. The volatile acid produced by this organism is acetic acid (11), and the initial levels in the medium came from the Trypticase which contains significant levels of acetate (Day and Costilow, *unpublished data*). It is likely that the two strains which used the acetate were oxidizing some acetate throughout the growth period since the rate and extent of acid accumulation in these cultures were much lower than with 2309S which failed to show any evidence of acetate utilization (Fig. 1). Also, the pH did not decline as rapidly in the cultures showing acetate utilization as in the culture of 2309S.

The changes in pH were even more pronounced when 2309MC was grown in TYG medium containing only 1 g of glucose per liter and without added potassium phosphate (Fig. 2). The pH of this culture decreased to about pH 6.7 at about the time the culture reached stationary phase of growth and remained fairly constant for several hours. At this point, the pH started to increase rapidly, and there was a marked increase in optical density indicating a growth response. The sharp increase in pH corresponded with the approximate time of glucose exhaustion from the

A Nuclear-Chicago Mark I scintillation spectrometer was used for all radioactivity counts. Where indicated, counts per minute were converted to disintegrations per minute after correcting for various efficiencies by using the channel-ratio procedure.

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**RESULTS**

**Fig. 1. Changes in pH and volatile acid concentration during growth of three strains of *B. popilliae* in buffered TYG medium. Solid lines are for pH and dashed lines for volatile acid concentration. Symbols: ○, 2309PA; ○, 2309S; and ▽, 2309M.**

**Fig. 2. Changes in optical density and pH and utilization of glucose during growth of two strains of *B. popilliae* in nonbuffered TYG medium. The solid lines are for strain 2309MC and the dashed lines for 2309M. Symbols: ○, optical density; ○, pH; and ▽, △, glucose concentration.**
A culture of strain 2309M derived from heat-shocked spores failed to exhibit an increase in pH or a secondary growth response. The pH declined to a level below 6.0 and remained low throughout the incubation period.

During the course of these studies, we encountered difficulty in obtaining the kind of pH response described above for 2309MC in unbuffered media although a pH rise was evident in TYG containing 4 g of K$_2$HPO$_4$ per liter. Since we were using yeast extract from a fresh shipment at that time, we tested the yeast extract from a number of different bottles with different control numbers. Of eight lots tested, only two resulted in the characteristic rise in pH shown in Fig. 2. However, a pH rise was evident in cultures of this strain with all yeast extracts tested, providing 4 g of K$_2$HPO$_4$ per liter was added. The final pH reached before exhaustion of the glucose may be of prime importance since previous data demonstrated that strain 2309PA oxidized acetate more rapidly at neutral pH than at lower levels (12). When the initial glucose concentration was increased from 1 to 2 g per liter in the unbuffered medium, the pH dropped to 6.0 to 6.3 in 2 days and failed to increase again.

**Oxidation of acetate by resting cells.** Cells of 2309MC harvested from the mid-log phase of growth (9-hr-old culture) oxidized acetate with a Q$_{O_2}$ [microliters of O$_2$ uptake per milligram (dry weight) per hour] of 9, whereas stationary phase cells (32-hr-old culture) of this strain oxidized acetate with a Q$_{O_2}$ of 17. The rates of oxygen uptake were constant during 2 hr of incubation with ~12 mg (dry weight) of cells in the Warburg vessel. Thus, the data indicate that this strain does oxidize acetate during exponential growth, but the rate of oxidation is much greater during the stationary phase of growth.

**B. popilliae** 2309MC also oxidizes glutamate, and the rate of oxidation of a mixture of glutamate and acetate is greater than the sum of the rates of oxidation of either substrate alone (Fig. 3). Such a result might be expected if glutamate was supplying four carbon intermediates of the tricarboxylic acid cycle to replace those used in the formation of glutamate and aspartate by the cells. As shown below, these two amino acids are formed from $[^{14}C]$acetate by resting cells. The rates of oxygen uptake by cells of this culture with succinate, malate, or fumarate were barely above the endogenous rate, and there was no significant stimulation of acetate oxidation by these compounds. This may have been due to the

**Table 2. Production of $^{14}$CO$_2$ from $[^{14}C]$acetate by various strains of Bacillus popilliae**

| Strain       | $[^{14}C]$ produced (counts/min) $[^{14}C]$acetate | $[^{14}C]$acetate |
|--------------|--------------------------------------------------|------------------|
|              | $[^{1-14}C]$ | $[^{2-14}C]$ |
| No spores formed in vitro | | |
| 2309         | 0.5             | 0.1             |
| 2309S        | 0.3             | 0.1             |
| 2309MC       | 73.6            | 58.5            |
| 2309N        | 88.5            | 69.0            |
| Spores formed in vitro | | |
| 2309M        | 3.5             | 2.0             |
| MS-54        | 8.5             | 5.4             |
| MS-69        | 38.6            | 38.6e           |
| MS-85        | 9.0             | 10.6            |

* Reaction mixtures of 3-ml volume in Warburg vessels contained 15 $\mu$moles of the $[^{14}C]$acetate indicated (45,400 counts per min per $\mu$ mole) and 10 to 15 mg (dry weight) of cells in 0.05 M potassium phosphate buffer. Cells were harvested from stationary phase cultures in Trypticase-yeast extract-glucose broth. They were incubated for 1 hr at 30 C.

* Values to be multiplied by $10^9$.

* Determined after several subcultures in Trypticase-yeast extract-glucose broth.
TABLE 3. Distribution of $^{14}$C from [1-$^{14}$C] acetate incorporated into various fractions of cells

| Cell fraction         | Cells (10-hr-old) Dpm*       | Per cent* | Cells (23-hr-old) Dpm*       | Per cent* |
|-----------------------|-------------------------------|-----------|-------------------------------|-----------|
| Cold trichloroacetate | 3.4                           | 40        | 41.6                          | 57        |
| Hot trichloroacetic   | 0.3                           | 4         | 2.5                           | 3         |
| Ethanol               | 2.3                           | 27        | 13.7                          | 19        |
| Residue               | 2.3                           | 27        | 9.9                           | 14        |

* Each Warburg flask contained 10 to 15 mg (dry weight) of cells, 20 $\mu$moles of acetate containing 0.83 $\mu$Ci of [1-$^{14}$C] acetate, and 0.05 M phosphate buffer at pH 7.4. The center well contained 0.2 ml of 20% KOH. The cells used were from stationary-phase cultures of 2309MC in Trypticase-yeast extract-glucose broth. Incorporation was determined after 60 min.

* Values to be multiplied by 10$^6$.

* Per cent of total $^{14}$C in cells.

pH (7.4) at which they were tested since previous data (12) demonstrated some oxidation of succinate and malate at pH 6.1 but essentially none at 7.4. Glyoxalate was not oxidized to a detectable extent.

All strains of B. popilliae tested except 2309S produced relatively low but significant levels of $^{14}$CO$_2$ from both [1-$^{14}$C] and [2-$^{14}$C]acetate during 1 hr of incubation (Table 2). However, the parent wild-type strain (2309) failed to produce significant levels of $^{14}$CO$_2$ until it had been cultivated in broth cultures through several transfers. Strain MS-54 also showed a much higher capacity to oxidize acetate after several subcultures in TYG broth. It is clear from these data that there is a wide variation among the strains of B. popilliae to oxidize acetate. Also, although all of the strains which we have found to be oligosporogenous in vitro do oxidize acetate, two strains (2309MC and 2309N) derived from 2309M no longer produce spores in vitro but have the highest capacity for acetate oxidation of those tested.

Incorporation of $^{14}$C from [1-$^{14}$C] acetate into cells and tricarboxylic acid cycle intermediates. Only a very small fraction (~2.5%) of the $^{14}$C from [1-$^{14}$C] acetate was incorporated into cells harvested from either 10-hr- or 23-hr-old cultures during a 60-min period. The 10-hr- and 23-hr-old cells oxidized 13 and 51%, respectively, of acetate present during 60 min of incubation, and between 97 and 98% of that oxidized by both lots of cells was recovered as $^{14}$CO$_2$. A high percentage of the $^{14}$C in the cells was recovered in the cold trichloroacetic acid fractions, but significant amounts were associated with the lipid (ethanol soluble) and protein (residue) fractions (Table 3). One would not expect a great amount of incorporation of acetate by resting cells of this organism since a number of amino acids are required for its growth (17).

Two-dimensional chromatography of the cold trichloroacetic acid extract from these cells on thin-layer plates revealed the presence of a number of $^{14}$C tricarboxylic acid cycle intermediates or related compounds (Fig. 4). There were highly significant amounts of $^{14}$C in spots corresponding to aspartate, citrate, glutamate, and succinate. The other counts are either too low or too closely associated with other radioactive spots to be of significance.

DISCUSSION

It appears likely that B. popilliae has the genetic potential for the oxidation of acetate, but that this activity is strongly repressed in the wild-type strain growing in vitro. The growth conditions influence greatly the acetate-oxidizing ability of the variants studied during this investigation. For example, the particular batch of yeast extract used in the medium had a pronounced effect. These factors may account for the difference between these results and those of Bulla et al. (4). The
latter investigators used strain 2309 and failed to observe any oxidation of acetate. We also failed to observe any activity of cultures of this strain started from spores, but we did observe some acetate oxidation after several subcultures in a broth medium.

Of particular interest is the fact that all variant cultures which produced spores at low frequencies in vitro also oxidized acetate. Three of these strains were derived from the wild-type 2309 by treatment with a mutagen. Thus, this ability may be one block to in vitro sporulation of wild-type cultures. However, this is obviously not the only block to spore formation in B. popilliae since other strains which did not produce spores were even more active in oxidizing acetate than those which did sporulate.

The data presented herein do not establish conclusively that a complete citric acid cycle is operative in some strains of B. popilliae. However, the evidence strongly indicates that it is. Thus, there is extensive oxidation of both [7-14C] and [2-14C]acetate; 14C from [7-14C]acetate is incorporated into citrate, glutamate, succinate, and aspartate; and the rate of oxygen uptake in the presence of acetate and glutamate is greater than the sum of the rates of uptakes with the individual substrates. These indications are fortified by previously reported data showing that (i) acetate was oxidized with a respiratory quotient of 1 (12), (ii) succinate and malate were oxidized slowly at low pH (12), and (iii) a condensing enzyme (12) and aconitase (9) were present. All attempts to detect α-ketoglutarate dehydrogenase have failed, but this may be due to a low specific activity of this enzyme. Also, all attempts to demonstrate the presence of a glyoxalate cycle have failed, and arsenite completely inhibits the oxidation of acetate (12). Therefore, this cycle is probably not operative in B. popilliae. This is further indicated by the increased rate of respiration observed in the presence of acetate and glutamate noted above.

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