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Medium Promoting Sporulation of *Bacillus larvae* and Metabolism of Medium Components

D. W. DINGMAN† AND DONALD P. STAHLY*

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242

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A new medium, designated TMYGP broth, was developed that allowed the honeybee pathogen *Bacillus larvae* NRRL B-3650 to produce up to 5×10^8 spores per ml of culture (microscopic count). This species normally sporulates poorly, if at all, in artificial broth media. An aeration rate lower than that normally used to cultivate other *Bacillus* species was required for sporulation. During the exponential growth phase, acids were produced by catabolism of yeast extract components, causing a decrease in pH of the medium. Thereafter, the pH began to increase, probably because of derepression of the citric acid cycle and consumption of the acids. Only after this time did usage of glucose from the medium occur. Thus, glucose usage seems to be regulated by catabolite repression. The presence of glucose was needed for one or more of the later events of sporulation. Of many substances tested, only gluconic acid and glucosamine partially substituted for glucose as a requirement for sporulation. Pyruvate was also required for good sporulation. It was metabolized during the late-exponential phase of growth.

Bacillus larvae is a pathogen of honeybee larvae, causing a fatal disease called American foulbrood. About 3% of all bee colonies inspected in the United States are infected with *B. larvae* (20). It was recognized early (22) that only spores are able to transmit the disease. The spores germinate in the midgut of the bee larvae, and the resulting vegetative cells invade the hemocoel, where they grow in the hemolymph to very large populations (1, 17, 18, 24). The cells sporulate efficiently in the hemolymph. Spores released from dead bee larvae may be spread to other larvae.

Whereas *B. larvae* sporulates efficiently in the hemolymph of bee larvae, most strains sporulate very poorly in or on artificial media. The study of foulbrood has been slowed somewhat by the difficulty of obtaining large numbers of spores in vitro. Some sporulation occurs on solid media (1, 8, 12, 16), but only two reports of fair-to-good sporulation in broth media exist. St. Julian and Bulla (21) obtained about 2×10^7 spores per ml after growth of *B. larvae* NRRL B-2605 in liquid MD medium, which contains yeast extract, glucose, and K_2HPO_4. Gochnauer (11) obtained 10^8 spores per ml after growth of *B. larvae* 34A in brain heart infusion broth plus thiamine hydrochloride. Other strains sporulated much less efficiently. The spore counts obtained in both of these studies were direct microscopic counts rather than counts of heat-resistant CFU.

Our interest in the topic of in vitro sporulation was sparked by our observation that several strains of *B. larvae* sporulated to some degree on plates of MYPGP agar, which contains yeast extract, Mueller-Hinton broth, glucose, K_2HPO_4, sodium pyruvate, and agar, but did not in broth cultures of the same medium without agar.

This paper describes the development of a liquid medium that supported good sporulation of *B. larvae* NRRL B-3650, the strain that sporulated the best on MYPGP agar. Results of experiments to determine when the components of the new medium (designated TMYGP broth) were used are also included. It was our hope that this information would provide clues as to why the medium components were required for sporulation. One of the surprising findings of this study was that glucose utilization occurred only after the apparent induction of the citric acid cycle at the end of the exponential phase of growth. Glucose was required by this strain during the stationary phase for good sporulation to occur. Another important discovery was that sporulation only occurred when the aeration rate was lower than that normally used for sporulation of other *Bacillus* species.

MATERIALS AND METHODS

Microorganisms. *B. larvae* NRRL B-3650 was the main organism used throughout this study. Other

† Present address: Department of Molecular Biology and Microbiology, School of Medicine, Tufts University, Boston, MA 02111.

strains of *B. larvae* used were NRRL B-2605, NRRL B-3553, NRRL B-3554, NRRL B-3555, and NRRL B-3558.

Media. MYPGP agar consisted of 1.5% yeast extract (Difco Laboratories), 1.0% Mueller-Hinton broth (Difco Laboratories), 0.2% glucose, 0.3% K_2HPO_4, 0.1% sodium pyruvate, and 2.0% agar. MYPGP broth was the same as above but without agar. TMYGP broth contained 1.5% yeast extract, 0.4% (22 mM) glucose, 0.1% (9.09 mM) sodium pyruvate, and 0.03 M Tris-maleate buffer (pH 7.0). MD broth, the medium used by Bulla et al. (2, 21), contained 1.5% yeast extract and 0.6% K_2HPO_4. The broth medium used by Gochnauer (11) was brain heart infusion broth (Difco Laboratories; 3.7%) supplemented with 10 μg of thiamine per ml. All components except glucose and thiamine were usually sterilized together by being autoclaved. Glucose and thiamine were added separately. An autoclaved 50% (wt/vol) stock solution of glucose and a filter-sterilized 1-mg/ml stock solution of thiamine were used.

Viable and spore counts. Viable counts were obtained by plating cells on the surface of MYPGP plates. Spore counts were obtained by direct microscopic counting with a Petroff-Hauser counting chamber or by plating on MYPGP plates after heating at 65°C for 15 min. *B. larvae* spores are noted for their low efficiency of germination (1). In our experience, the heat-resistant spore counts were usually about 6% of the direct microscopic spore counts.

Cultural conditions. Unless specified otherwise, broth cultures were grown in 6 ml of medium in loosely capped test tubes (20 by 150 mm) at 37°C in a rotary incubator shaker (New Brunswick Scientific Co.) adjusted to 195 rpm. The tubes were held at a 45° angle in test tube racks. The turbidity of broth cultures was measured with a Klett-Summerson photoelectric colorimeter with a no. 54 filter. Plates were incubated at 37°C for at least 4 days before colonies were counted. Results of plate counts were expressed as CFU per milliliter of culture.

Glucose and pyruvate assays. Glucostat Special (Worthington Diagnostics) was used to measure the concentration of glucose. Pyruvate concentration was measured by the method of Von Korff (23), using lactate dehydrogenase (Sigma Chemical Co.).

RESULTS

Development of TMYGP broth medium. Good sporulation of *B. larvae* NRRL B-3650 occurred on the surface of MYPGP agar but not in a broth medium that was identical except for the omission of agar. Omission of individual components of the MYPGP broth medium led to the discovery that Mueller-Hinton broth inhibited sporulation.

Experiments were next performed in which the concentration of each remaining component of MYPGP broth was varied while the concentrations of other components were held constant (Table 1). (The K_2HPO_4 of MYPGP broth was replaced by 0.03 M Tris-maleate buffer [pH 7.0] because of an unexplainable irreproducibility in the extent of sporulation when K_2HPO_4 was used.) Glucose was required for sporulation. More than 10^7 spores per ml of culture were formed when the glucose concentration was between 16.7 and 55 mM (the highest concentration tested). Heat-resistant spore counts corresponded with direct microscopic spore counts of about 17 times higher (see Materials and Methods). Yeast extract was required for growth. Efficient sporulation ($\geq 10^7$ spores per ml of culture) occurred only in the presence of 1.5 to 2.25% yeast extract. Failure to sporulate at high yeast extract concentrations probably was, at least in part, due to excessive acid accumulation

TABLE 1. Effect of concentration of medium components on extent of sporulation[a]

Component concn	No. of spores (CFU/ml)
Glucose (mM)	
0	$<1.0 \times 10$
5.55	5.7×10^4
11.1	9.5×10^5
16.7	1.9×10^7
22.2	4.5×10^7
27.8	2.0×10^7
33.3	5.4×10^7
44.4	1.9×10^7
55.5	1.4×10^7
Yeast extract (%)	
0[b]	$<1.0 \times 10$
0.75	5.1×10^2
1.5	4.5×10^7
2.25	1.5×10^7
3.0	$<1.0 \times 10$
3.75	$<1.0 \times 10$
4.5	$<1.0 \times 10$
Sodium pyruvate (mM)	
0	4.1×10^2
4.54	4.1×10^7
9.09	4.5×10^7
13.6	2.3×10^7
18.18	2.2×10^7
22.72	2.0×10^7
27.27	2.1×10^7
Tris-maleate (M)	
0	3.0×10
0.01	1.4×10^7
0.02	3.9×10^7
0.03	4.5×10^7
0.045	7.8×10^6
0.06	2.7×10^3
0.075	$<1.0 \times 10$
0.09	$<1.0 \times 10$

[a] The concentrations of separate components were varied while the concentrations of all other components were held constant and equal to that of TMYGP medium. All cultures were incubated for 4 days before the number of heat-resistant spores was determined.

[b] No growth occurred in the absence of yeast extract.

TABLE 2. Effect of volume of medium on extent of sporulation[a]

Vol of TMYGP medium (ml)	No. of spores (CFU/ml)
2	8.0×10
3	$<1.0 \times 10$
4	3.4×10^5
5	3.7×10^7
6	4.5×10^7
8	2.5×10^7
10	1.0×10^7
12	5.0×10^7
15	1.3×10^7
20	$<1.0 \times 10$

[a] All cultures were incubated for 4 days before the number of heat-resistant spores was determined.

in the medium. When the medium was adjusted to pH 7.3 rather than 6.9 (the pH of TMYGP medium without pH adjustment), 6.8×10^7 spores per ml of culture occurred in medium containing 3% yeast extract (see legend to Fig. 2). Sodium pyruvate concentrations of 4.54 mM and higher allowed good sporulation ($>10^7$ spores per ml). With some experiments, the requirement for pyruvate was not as definite as indicated in Table 1. For example, 2×10^5 spores per ml were formed in the absence of pyruvate in one experiment (see Fig. 6, pyruvate). More than 10^7 spores per ml of culture occurred when the Tris-maleate buffer (pH 7.0) concentration was between 0.01 and 0.03 M. The composition of TMYGP medium (see Materials and Methods) was based upon the results shown in Table 1.

The effect of aeration on sporulation was tested by incubating different volumes of culture in test tubes on a rotary shaker. As shown in Table 2, the level of aeration was critical for sporulation. More than 10^7 spores per ml of culture were produced only when the volume was between 5 and 15 ml. The effect of aeration was primarily on sporulation since good growth occurred in all samples. (The generation time was 1.6 h for all of the cultures.) Some evaporation occurs, of course, during 4 days of incubation. Essentially the same results were obtained when the volume of each tube was kept constant by periodic addition of sterile water.

Growth, sporulation, and metabolism of components of TMYGP broth by *B. larvae* NRRL B-3650. Figure 1A shows the pattern of growth and sporulation of strain B-3650 in TMYGP medium. Growth was measured by both viable count and turbidity. Also shown (Fig. 1B) are pH of the medium and concentrations of glucose and pyruvate in the medium as functions of culture age. During the exponential phase of growth, cells were in chains of four to six cells. By 23 h, which was 5 h after attainment of maximum turbidity, considerable lysis was observed microscopically, accompanied by a slight decline in turbidity. Simultaneously, the chains broke into individual cells. No decrease in viable count was observed at this time; in fact, a slight increase occurred. This increase in viable count was considered an artifact due to cell separation. Cultural turbidity is probably a more reliable measure of growth. Heat-resistant spores began to appear at about 39 h and reached a maximum at 55 h. The time of initial increase is not shown in Fig. 1A since the counts were $<10^6$/ml. Percent sporulation in the experiment described in Fig. 1 was calculated to be 68% based on numbers of spores (1.8×10^7 heat-resistant cells per ml times 17) and the maximum viable cell count (4.5×10^8 CFU/ml). As indicated above, there was uncertainty concerning the maximum viable count because of association of the cells in chains. The pH of the medium declined during growth, reaching a minimum at the end of the exponential phase. Thereafter, it increased to a value of about 7.7, much higher than the pH of uninoculated medium (6.9).

With most *Bacillus* species previously studied (19) the decline in pH is due to acid accumulation in the medium that resulted from catabolism of glucose. The increase in pH is usually due to complete oxidation of the acids, which results from derepression of the tricarboxylic acid (TCA) cycle upon exhaustion of glucose from the medium. During growth of *B. larvae* B-3650, acids accumulated because of incomplete oxidation of substances in the yeast extract. Figure 2A illustrates the effect of various yeast extract concentrations in TMYGP medium on growth and pH profile. The decrease in pH was proportional to the yeast extract concentration. Figure 2B shows again that glucose usage only began after the pH minimum was attained. The increase in time at which the increase in pH occurred as the yeast extract concentration was increased implies that some component of yeast extract has to be depleted before derepression of the TCA cycle occurs.

It is theoretically possible that pyruvate exhaustion permits derepression of the TCA cycle. Pyruvate was metabolized during growth, disappearing from the medium at or before the pH minimum (Fig. 1B and 2B). We do not favor this hypothesis, however, since growth in medium consisting only of yeast extract also exhibited a typical pH profile (data not shown). (For the pH change that occurred in medium consisting of only buffer plus yeast extract, see Fig. 4A.) The role of pyruvate in sporulation is discussed further below.

Further evidence substantiating the contention that glucose metabolism is not responsible

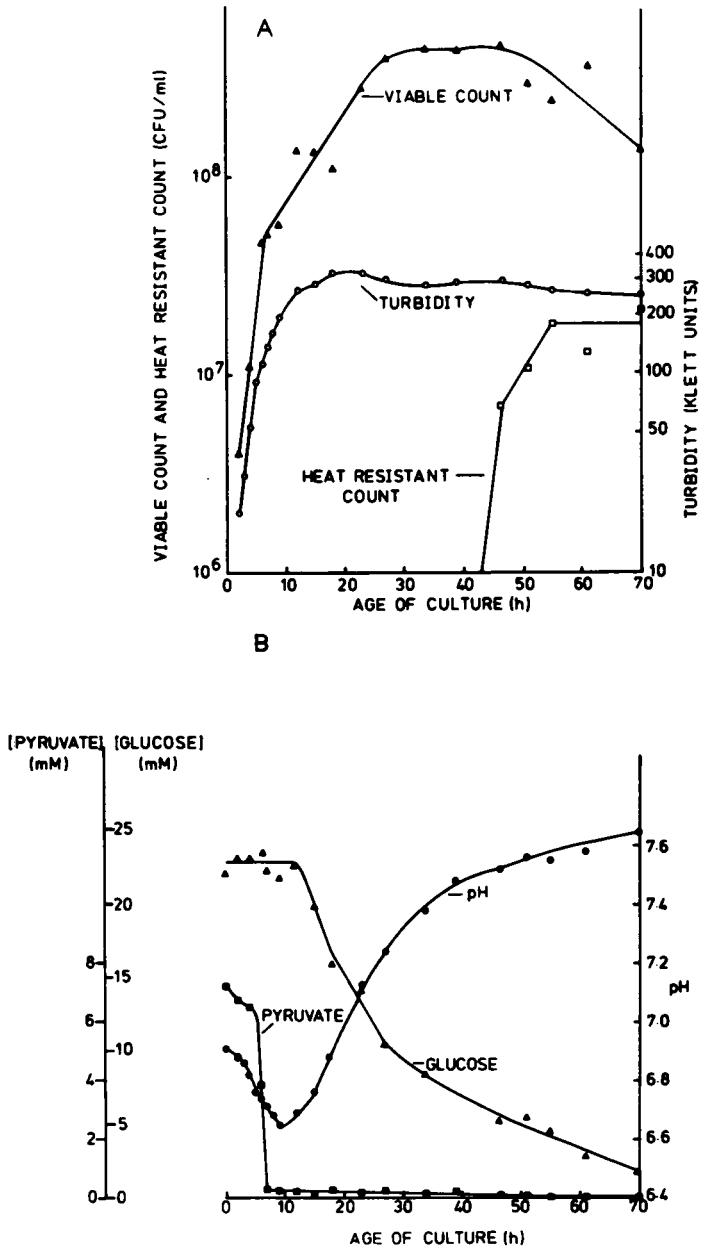

FIG. 1. Growth and sporulation of *B. larvae* B-3650 in TMYGP medium. (A) Viable count (△), turbidity (○), and heat-resistant spore count (□) as functions of culture age. (B) pH of the medium (●), glucose concentration of the medium (▲), and pyruvate concentration of the medium (■) as functions of culture age.

for the drop in pH that occurs during growth was provided by an experiment in which turbidity, pH of the medium, and glucose concentration of the medium were measured at different stages of development in cultures grown in TMYGP medium with and without glucose (Fig. 3). The turbidity curves show that the growth rates were equivalent in the two cultures, but at about 8.5 to 9 h, the turbidity curves diverged, with an increase in turbidity occurring only in the glucose-containing culture. This coincided with the beginning of glucose utilization. The pH increased in both cultures, but the rate of increase was greater in the culture without glucose. Whereas glucose metabolism was not responsible for the initial decline in pH, its use after the

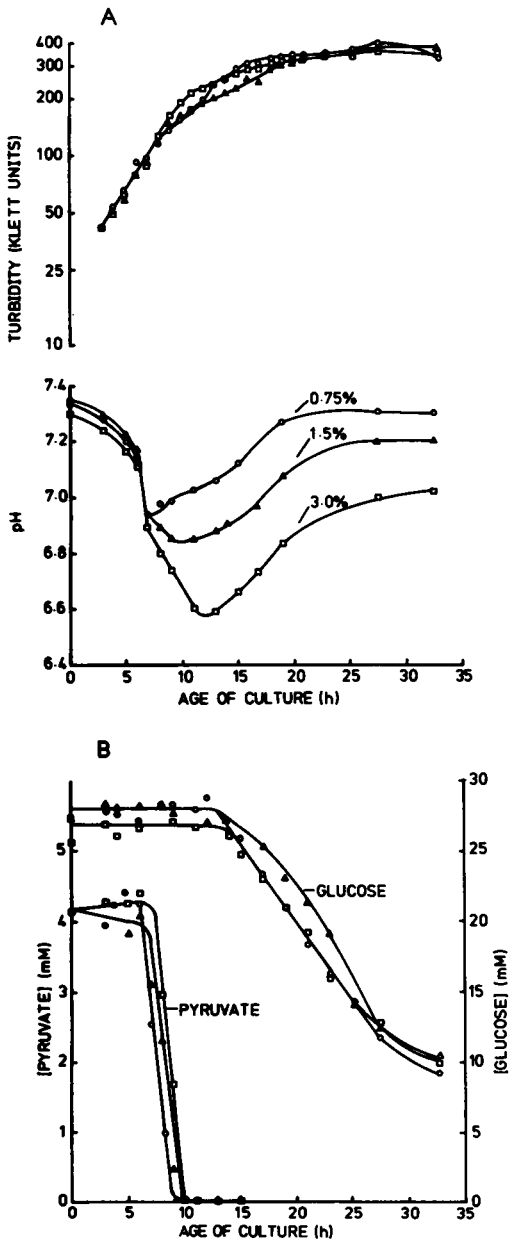

FIG. 2. Effect of yeast extract concentration of TMYGP medium on growth and sporulation. The medium was adjusted to pH 7.3 before being autoclaved. (A) Turbidity and pH of the medium. (B) Glucose and pyruvate concentrations of the medium. Yeast extract concentrations were 0.75% (○), 1.5% (the normal concentration) (△), and 3.0% (□). Spore counts after 4 days of incubation in media containing 0.75, 1.5, and 3.0% yeast extract were 1.5×10^6, 2.8×10^7, and 6.8×10^7/ml, respectively.

pH minimum is reached may result in some acid production. Alternatively, or in addition, glucose metabolism may cause partial repression of

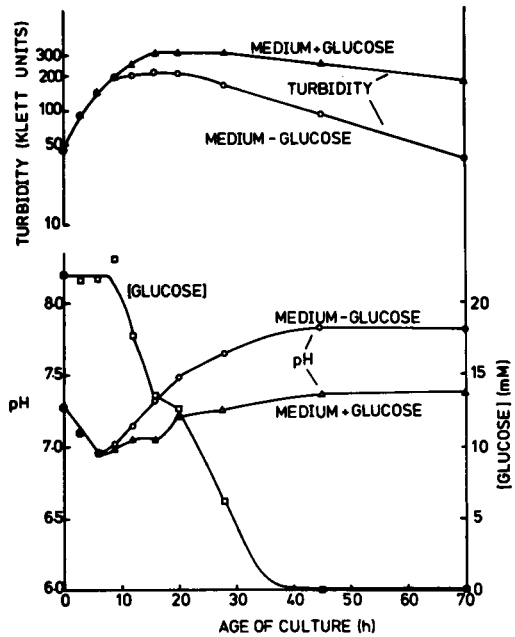

FIG. 3. Turbidity, pH of the medium, and glucose concentration of the medium as functions of culture age in complete TMYGP medium and TMYGP medium lacking glucose. Symbols: (△) turbidity and pH of the medium in complete TMYGP medium; (○) turbidity and pH of the medium in TMYGP medium lacking glucose; (□) glucose concentration of complete TMYGP medium.

the TCA cycle, thus slowing the increase in pH.

The effect of pyruvate on turbidity, pH of the medium, and pattern of glucose utilization is shown in Fig. 4. Basal medium consisted of Tris-maleate buffer and yeast extract. Cultures were grown in basal medium containing the following additional nutrients: (i) none, (ii) pyruvate, (iii) glucose, and (iv) pyruvate plus glucose (complete TMYGP medium). Significant sporulation occurred only in complete TMYGP medium. The cultures all grew at the same rate, but the cultures containing pyruvate exhibited more lysis (beginning at about 15 h). Almost complete lysis occurred by 69 h in medium ii. The pH rise occurred more rapidly in medium iv, which contained glucose and pyruvate, than in medium iii, which contained no pyruvate. It is possible that pyruvate (or a pyruvate catabolite) stimulates the derepression of the TCA cycle enzymes or enhances activity of the enzymes. Alternatively, or, in addition, pyruvate (or a pyruvate catabolite) may stimulate induction of amino acid decarboxylases or deaminases responsible for production of basic products (amines or ammonium hydroxide). The latter hypothesis is suggested by the much higher pH attained during the stationary phase in basal medium plus

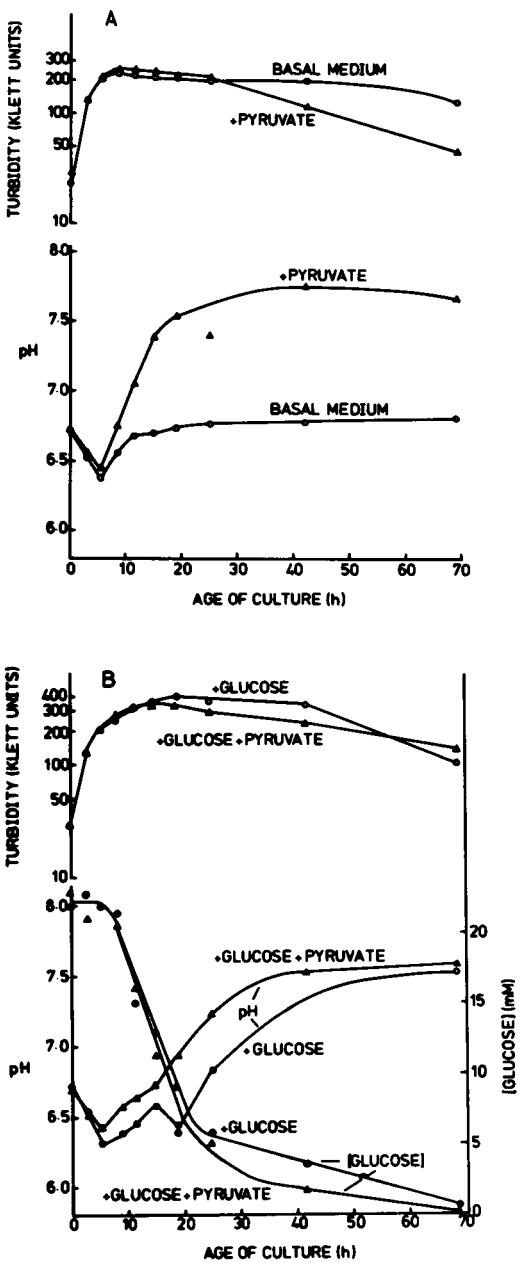

FIG. 4. Parameters associated with growth and sporulation in basal medium (Tris-maleate and yeast extract) and basal medium to which pyruvate, glucose, and pyruvate plus glucose have been added. (A) pH and turbidity in basal medium (○), and pH and turbidity in basal medium to which pyruvate was added (△). (B) pH and turbidity in basal medium to which glucose was added (○), pH and turbidity in basal medium to which glucose and pyruvate were added (complete TMYGP medium) (△), and glucose concentrations in the above two media (● and ▲, respectively).

pyruvate compared with that in basal medium (Fig. 4A). Pyruvate was not required for attainment of the high pH when glucose was included in the medium (Fig. 4B).

Pyruvate is one of the acids that accumulated in TMYGP medium from which pyruvate was omitted. Figure 5 shows that about 0.28 mM pyruvate accumulated during growth. Its utilization was complete before the pH minimum was attained, an observation in agreement with that noted for pyruvate-supplemented media (Fig. 1B and 2B). The nature of other acids that accumulate in the medium is not known.

Effect of time of addition of nutrients on efficiency of sporulation. It is clear from the preceding discussion that glucose, pyruvate, and yeast extract are required for sporulation. An experiment was performed to determine at what stage of development each of these substances had to be present for efficient sporulation to occur. As mentioned previously, only yeast extract is required for growth. Three different batches of medium were made, one lacking pyruvate, one lacking glucose, and one containing one-fourth the normal concentration of yeast extract. To separate portions of each medium, the missing nutrient was added to the normal concentration at 11 different times during growth and sporulation. Spores were first observed microscopically after about 45 h. After sporulation was complete, spore counts were made. The results in Fig. 6 indicate that glucose could be added as late as 35 h for efficient sporulation to occur. If the full complement of yeast extract was added after 16 h (early stationary phase), a reduction in extent of sporulation occurred. Pyruvate was needed during the growth phase for good sporulation to occur. Addition after 8 h resulted in a decrease in spore titer.

Substitutes for glucose and pyruvate in TMYGP broth. An experiment was performed to ascertain whether other carbon sources could substitute for glucose as a nutrient required for sporulation (Table 3). Of the substances tested other than glucose, only sodium gluconate and glucosamine allowed fair sporulation ($>10^6$ spores per ml of culture).

A similar experiment was performed to test potential replacements for sodium pyruvate. None of the following substances at a 9 mM final concentration permitted sporulation when substituted for sodium pyruvate: ammonium lactate, sodium acetate, sodium citrate, potassium glutamate, and sodium succinate. In one experiment, all gave spore titers of <10 CFU/ml compared with 1.9×10^7 CFU/ml in the pyruvate-containing culture.

Comparison of glucose catabolic patterns of *B. larvae* B-3650 and B-2605. As stated previously, glucose catabolism in strain B-3650 began after

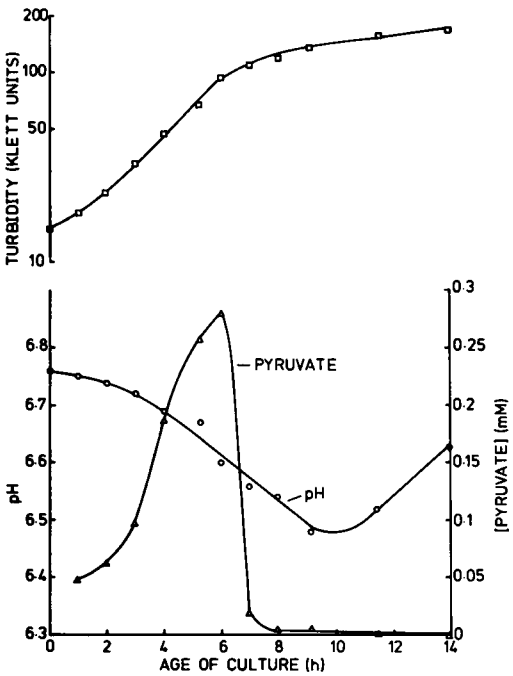

FIG. 5. Accumulation of pyruvate during growth in TMYGP broth from which pyruvate was omitted. Symbols: (○) pH of the medium; (△) pyruvate concentration of the medium; (□) culture turbidity.

the beginning of the pH increase of the medium. St. Julian and Bulla (21) reported that a very different situation occurs with *B. larvae* NRRL B-2605. With this strain, glucose catabolism occurs before the pH minimum. The glucose is incompletely oxidized to acidic end products, and the pH of the medium does not increase until glucose is exhausted. The medium used by these investigators was MD broth (see Materials and Methods). Figure 7 shows an experiment in which both *B. larvae* strains were grown in MD broth. The patterns of turbidity, pH, and glucose used by strain B-3650 were almost the same in MD broth as in TMYGP broth (Fig. 7A). However, the results with strain B-2605 were very different and were the same as those reported by St. Julian and Bulla (21). There was no apparent derepression of the TCA cycle until glucose was depleted from the medium (Fig. 7B). Similar results were obtained when strain B-2605 was grown in TMYGP broth (data not shown).

Sporulation of *B. larvae* B-3650 in other media and sporulation of other strains in TMYGP broth. The maximum spore counts per milliliter of culture for *B. larvae* B-3650 in the MD medium described by St. Julian and Bulla (21) and in the brain heart infusion broth described by Gochnauer (11) were about 5 and 0.004%, respectively, of that obtained in TMYGP broth. Thus, TMYGP medium is the best medium for sporulation of this strain.

Unfortunately, TMYGP broth does not support good sporulation of several other strains of *B. larvae*. The maximum heat-resistant spore counts (in CFU per milliliter of culture) for strains B-2605, B-3553, B-3554, B-3555, and B-3558 were <10, <10, 2.6×10^3, <10, and <10, respectively.

DISCUSSION

A medium was devised that permitted good sporulation of *B. larvae* NRRL B-3650. It contained yeast extract, glucose, pyruvate, and Tris-maleate buffer (pH 7.0). Yeast extract was the only one of these nutrients that was required for growth; the glucose and pyruvate were required for sporulation.

In *B. larvae* B-3650, acids accumulated because of incomplete oxidation of components of yeast extract during growth. The nature of the acids generated by catabolism of yeast extract components (probably amino acids) is not known except for the small amount of pyruvate (Fig. 5). Amino acid catabolism in most orga-

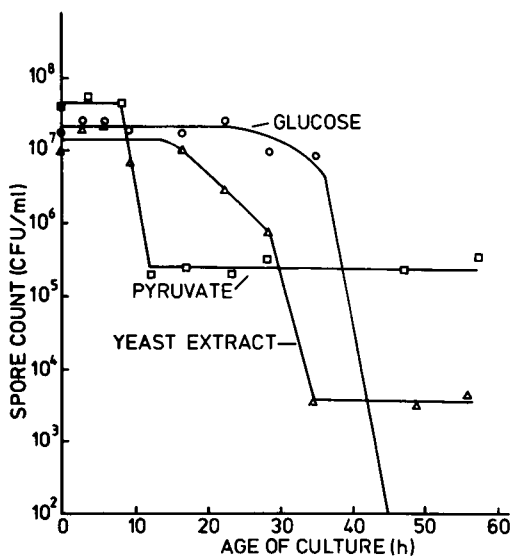

FIG. 6. Effect of time of addition of glucose, pyruvate, and yeast extract on extent of sporulation. Symbols: (○) glucose addition; (△) yeast extract addition; (□) pyruvate addition. Basal media for the glucose and pyruvate addition experiments consisted of TMYGP medium from which the specified nutrient was omitted. In the case of yeast extract, one-fourth the normal concentration was included. At the specified times, glucose, pyruvate, and yeast extract were added so that the final concentrations were identical to that of TMYGP medium. Incubation was continued for 5 days, after which spore counts were made.

TABLE 3. Effect of carbon source replacements for glucose on extent of sporulation in TMYGP medium[a]

Carbon source	Final concn (mM)	No. of spores (CFU/ml)
None		$<1.0 \times 10$
Glucose	22.2	5.2×10^7
Potassium gluconate	22.2	1.4×10^6
Glucosamine	22.2	6.2×10^6
Galactose	22.2	$<1.0 \times 10$
Fructose	22.2	$<1.0 \times 10$
Mannitol	22.2	$<1.0 \times 10$
Glycerol	44.4	$<1.0 \times 10$
Sodium pyruvate	44.4	$<1.0 \times 10$
Sodium acetate	66.6	$<1.0 \times 10$
Sodium citrate	22.2	$<1.0 \times 10$
Potassium glutamate	44.4	1.1×10^3
Sodium succinate	44.4	$<1.0 \times 10$

[a] Basal medium consisted of yeast extract (1.5%), sodium pyruvate (9.09 mM), and Tris-maleate buffer (pH 7.0; 0.03 M). Each of the carbon sources was added to basal medium as a sterile stock (glucosamine and sodium pyruvate were filter sterilized; others were autoclaved). Spore titers were determined after incubation for 4 days.

nisms results in the production of pyruvate, acetate, or acids of the TCA cycle.

One of the requirements for sporulation of *B. larvae* is that nutritional and environmental conditions promote the derepression of the TCA cycle at the end of the exponential phase of growth. St. Julian and Bulla (21) demonstrated by radiorespirometric studies that *B. larvae* cells contained active TCA and glyoxylic acid pathways after the increase in pH occurred. *B. subtilis* mutants that are blocked in the TCA cycle, when cultivated under normal conditions, grow well vegetatively but are unable to sporulate (6, 14, 25). Many of the enzymes of this cycle are subject to apparent catabolite repression (4, 7, 13, 15). Glucose alone causes partial repression of one of the enzymes, aconitase, but complete repression requires the presence of both glucose and α-ketoglutarate-glutamate (5, 13).

In our study, yeast extract, when supplied in excess to *B. larvae* B-3650, prevented the increase in pH. We infer from this finding that yeast extract probably represses synthesis of TCA cycle enzymes. Enzymatic data are required to confirm this hypothesis, of course. The time of the assumed derepression of the TCA cycle is uncertain. The pyruvate disappearance from the medium occurs before the pH minimum is attained. Pyruvate may be funneled directly into the TCA cycle or it may be converted into some other compound; e.g., acetate or lactate. It is not converted into acetoin as it is in some other *Bacillus* species (12).

We found that glucose utilization began after the beginning of the pH increase of the medium. St. Julian and Bulla (21) reported that a very different situation occurs with *B. larvae* NRRL B-2605. With this strain, grown in MD medium,

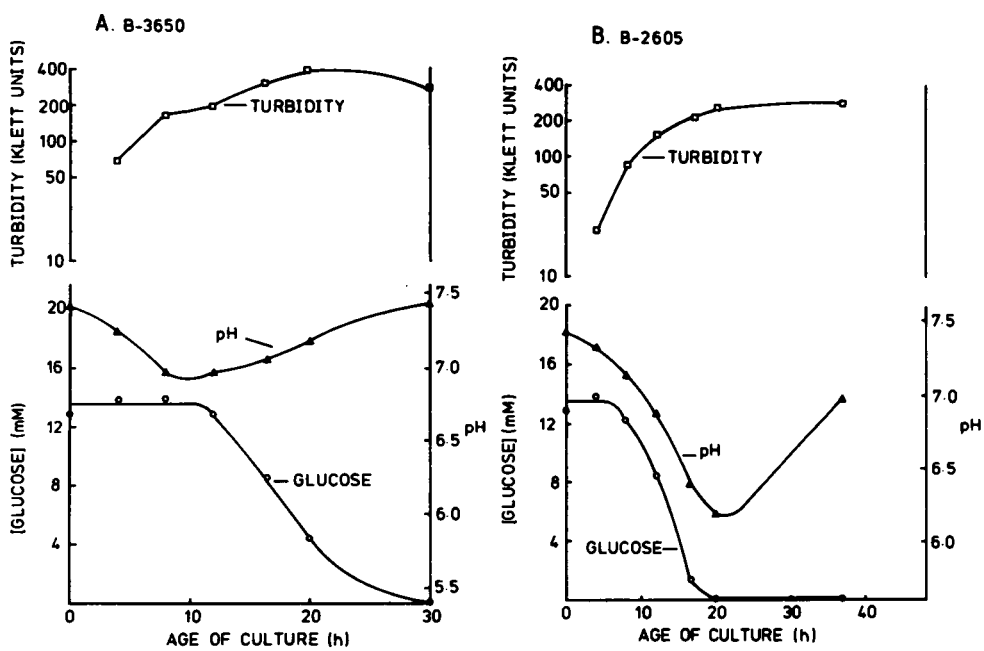

FIG. 7. Turbidity, pH of the medium, and glucose concentration of the medium as a function of culture age for *B. larvae* NRRL B-3650 and B-2605 growing in MD medium.

glucose metabolism during growth gives rise to acid products. The TCA cycle is not derepressed until the glucose is depleted. This difference in results was not due to different media. We grew both strains in both media and can clearly conclude that the difference in data was due to strain differences. Perhaps repression of the TCA cycle in *B. larvae* requires both catabolites derived from yeast extract and glucose. (It cannot be ruled out that a very small amount of glucose was used by strain B-3650 during the early growth phase.) It is possible that the yeast extract component is depleted more rapidly than glucose in strain B-3650 and that the opposite situation occurs in strain B-2605. This hypothesis is purely speculative, of course, and many other hypotheses are possible.

It appears that glucose utilization is controlled by catabolite repression in *B. larvae*. This repression could be exerted at the level of glucose transport or metabolism. The delay in glucose usage is not restricted to strain B-3650 (unpublished data). To our knowledge, this is the first report of apparent catabolite repression of glucose utilization during growth of *Bacillus* species.

Glucose was needed for one or more of the later events of sporulation in *B. larvae*; it could be added as late as 35 h for efficient sporulation to occur. The sporulation process begins at about 10 to 12 h. One possibility is that glucose is needed as an energy source since other energy sources may be depleted from the medium by this time. Alternatively, it could be that there is a defect in gluconeogenesis late in sporulation. Gluconeogenesis is required for sporulation of *B. subtilis* in a medium lacking glucose. It is required for production of cell membrane (from glycerol phosphate) and for synthesis of the peptidoglycan components of the germ cell wall and spore cortex (from glucosamine) (3, 9, 10). Sodium gluconate and glucosamine were the only carbon sources of many tested (Table 3) that could replace glucose as a nutrient required for sporulation of *B. larvae*. It is not known how sporulating cells of *B. larvae* metabolize gluconate, but vegetative cells metabolize glucose (and probably gluconate) predominantly via the pentose phosphate oxidative pathway (21). Fructose 6-phosphate could be generated from pentoses by operation of the transketolase-transaldolase mechanism and, hence, could be available for synthesis of the glucosamine and muramic acid components of peptidoglycan. Glycerol, pyruvate, acetate, and several acids of the TCA cycle were unable to replace glucose as a requirement for sporulation.

The pyruvate requirement for sporulation is intriguing, in part because it must be present during growth. The latest that it could be added was 8 h (Fig. 6), shortly before derepression of the TCA cycle. Cultures containing pyruvate showed a more rapid rise in pH (Fig. 4), a factor explainable by a more active TCA cycle or more active amino acid catabolism, the latter generating amines or ammonium hydroxide or both. Pyruvate itself could not be the cause of this stimulation since it was depleted from the medium before the increase in pH occurred (Fig. 1). Perhaps a product of pyruvate metabolism exerts the effect. Acetate and several acids of the TCA cycle were unable to substitute for pyruvate.

Maintenance of the proper amount of aeration was very important for sporulation. The vigorous aeration normally used for growth of more aerobic *Bacillus* species had no deleterious effect on the growth rate of *B. larvae*, but it almost totally prevented sporulation. It is not known whether excessive aeration prevents sporulation because of oxygen toxicity or because of removal of required CO_2. Oxygen toxicity is a possibility since *B. larvae* has 2,700 to 5,400 times less catalase than does *B. subtilis* and no apparent NADH peroxidase (Dingman and Stahly, manuscript in preparation).

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