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Sporulation of the "Thermophilic Anaerobes"

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A reasonable degree of synchrony in the sporulation of *Clostridium thermosaccharolyticum* 3814 was obtained by using three 10% transfers of 8-hr cultures in a medium containing 0.5% L-arabinose, 0.5% peptone, 0.5% yeast extract, and Gc minerals. Sporulation was stimulated by L-arabinose and L-xylose, but was repressed by glucose, mannose, fructose, and D-pentoses. Sporulating cells were long and thin, whereas repressed cells were shorter and thicker. The optimal *p*H for sporulation was in the range of *p*H 5.0 to 5.5. As sporulation continued, the accumulated acetate decreased. Label studies indicated that a significant amount of acetate-*2-C*^{14} was incorporated into the spore lipid. The calcium, phosphorus, and dipicolinic acid (DPA) concentrations on a dry weight basis were 2.55, 2.60, and 7.25%, respectively. The molar ratio of Ca-DPA was 1.47.

The general practice in producing spores of the "thermophilic anaerobes" is to employ meat or vegetable infusions containing tissue particles. Spores formed in these complex media are usually thermo-resistant, but they cannot be easily separated from the residual sporangia, vegetative cells, or the microscopic particles present in the infusions. The complexity of these media is generally unsatisfactory for physiological studies on the organism during vegetative growth and sporulation. Although solid media have been used for the production of spores (16, 21), it is difficult to study the changes affecting sporogenesis and optimal spore maturity in such a growth system. It is preferable to carry out sporulation studies in a nonparticulate liquid medium which permits frequent removal of representative samples for observations during growth and sporulation, without disruption of anaerobiosis. Some aspects of the nutritional and environmental conditions for the sporulation of the "thermophilic anaerobes" in a particulate pea broth have already been reported (C. G. Pheil, C. K. Lee, and Z. J. Ordal, Bacteriol. Proc., p. 17, 1966; C. G. Pheil, Ph.D. Thesis, Univ. of Illinois, Urbana, 1967). The present studies describe the effect of various carbohydrates on sporulation and provide data on the chemical characteristics of the resulting spores.

Materials and Methods

Test organisms. The major test organism used in these studies was *Clostridium thermosaccharolyticum*, National Canners Association (NCA) strain 3814, which was obtained from George York, University of California, Davis. The significant sporulation results were verified by use of "thermophilic anaerobes" isolated from canned foods which exhibited thermophilic "hard swell" type of spoilage. The TA-37 culture was obtained from the H. J. Heinz Co., and TA-3 and TA-4 were isolated in our laboratory. The stock cultures of these strains were stored at 3 C in pea broth (5) stratified with sterile Vaspar (10:1, petrolatum-mineral oil). The stock suspensions were transferred at approximately 3-month intervals into fresh pea broth.

Media. The basal sporulation medium used in all cases has been labeled "Gc" broth, and represents a modification of the "Gb" medium of Pelcher et al. (14). It has the following composition: peptone (Difco), 0.5%; yeast extract (Difco), 0.5%; $CaCl_2 \cdot 2H_2O$, 0.01%; $(NH_4)_2SO_4$, 0.1%; $MgSO_4$, 0.01%; $MnSO_4 \cdot H_2O$, 0.01%; $ZnSO_4 \cdot 7H_2O$, 0.0005%; $CuSO_4 \cdot 5H_2O$, 0.0005%; $MoSO_4$, 0.0001%; $FeSO_4 \cdot 7H_2O$, 0.00005%; and L-arabinose, 0.5%. A solution of the mineral salts was prepared in a concentration 10 times that required in the medium. The broth was prepared by adding the yeast extract and peptone to the diluted mineral salt solution and adjusting the *p*H to 6.8. The carbohydrates were filter-sterilized and added aseptically to each flask at the time of inoculation. In all sporulation studies, the medium was used immediately after sterilization and tempering to 58 C.

Modified pea extract-agar was used for viable-cell counts. The agar was prepared according to the procedure of Folinazzo and Troy (5), except that 1% agar was added.

Spore production. The active culture technique of Collier (3), with suitable modifications, was routinely used. Essentially, the method involved the activation of the inoculum by three successive transfers. The

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TABLE 1. *Effect of various carbohydrates on the sporulation of Clostridium thermosaccharolyticum 3814*[a]

Carbohydrate	Concn (%)	Final *p*H	Sporulation[b]
D-Glucose	0.5	4.28	<1
D-Glucose	0.1	5.35	<1
D-Mannose	0.5	5.00	<1
D-Galactose	0.5	5.03	<1
D-Fructose	0.5	4.89	<1
D-Arabinose	1.0	5.79	<1
D-Arabinose	0.5	5.80	<1
D-Ribose	0.5	5.42	<1
D-Xylose	0.5	6.14	<1
L-Xylose	0.5	5.32	22
L-Arabinose	0.5	5.42	32
L-Arabinose	0.1	6.00	<1
None	—	6.43	No growth

[a] Basal media contained 0.5% peptone, 0.5% yeast extract, and Gc minerals.

[b] Per cent sporulation was determined with a Petroff-Hausser counter.

initial culture in this series was inoculated with ca. 10^6 cells from a stock culture and was incubated at 58 C until maximal cell population was obtained. The cells were then transferred twice at a 10% inoculum level, at 8-hr intervals. The last transfer was incubated at 58 C for 24 hr, or until maximal spore production was obtained. To minimize oxygen reabsorption during inoculation, the medium was continuously gassed with a mixture of 10% H_2, 10% CO_2, and 80% N_2, which was passed over a Deoxy catalyst (Engelhard Industries, Newark, N.J.). Prior to incubation, the tubes or flasks were then stratified with sterile Vaspar.

The utilization of an initial vegetative-cell inoculum rather than a spore inoculum was necessary since heat-shocked spores exhibited an 18- to 24-hr lag prior to active vegetative-cell multiplication, regardless of the carbon source in the medium. These results support the observations of others (16, 20) in which a 2- to 3-week incubation period was required to obtain sporulation of the "thermophilic anaerobes."

The spores were separated from the sporangia by a short ultrasonic treatment (22 kc for 1 min at 5 C). This treatment released all spores from the sporangia and had no significant effect on the viable counts or heat resistance of the spores. The spores were separated from the residual vegetative cells and debris by several slow-speed centrifugations (9).

Total and viable counts. Total counts of vegetative cells and spores were made with a Petroff-Hausser counter.

The viable-spore populations were determined after a heat shock at 100 C for 10 min and by plating in modified pea extract-agar by use of the "agar deep" procedure in screw-cap test tubes (16 by 150 mm). The agar concentration in the pea extract medium had a definite effect on spore recovery. The use of 1% agar increased the spore recovery 10 times over that in media containing 2% agar. The lower spore recovery in 2% agar was in part due to excessive splitting of the agar by the large amounts of gas; in fact, isolated splitting of the agar was observed prior to colony formation. Increased spore recovery was also observed when 0.05% cysteine and 0.02% sodium sulfide was used instead of 0.2% sodium thioglycolate for maintaining anaerobiosis.

Analytical procedures. Spore hydrolysates were prepared by autoclaving the samples in 0.5 N HCl for 1 hr at 121 C. Calcium was determined by an ethylenediaminetetraacetic acid (EDTA) titration as described by Pelcher et al. (14). Pretreatment of the hydrolysates with 0.1 N potassium cyanide in 1 N sodium hydroxide removed the interfering iron.

Dipicolinic acid (DPA) was determined according to a modification of the colorimetric procedure of Jannsen et al. (8). A solution of DPA (Aldrich Chemical Co., Inc., Milwaukee, Wis.) of known concentration was used as the test standard.

The acetic acid concentration during growth and sporulation was determined in an Aerograph Hi Fy gas chromatograph with a flame detector. A 5-ft (152-cm) stainless-steel column coated with Carbowax 1540 (20% on an acid-washed 30/60 firebrick support) was used for the detection of the acetic acid in the aqueous samples. The injection and oven temperatures were 170 and 95 C, respectively.

The incorporation of acetate-*2-^{14}C* into cell components was determined by adding the labeled acetate to growing cells. After various periods of time, samples were removed from the culture, placed in a coolant bath at −10 C to stop incorporation, and cooled to 0 to 4 C. Cell fractionation was carried out as described by Roberts et al. (17). The radioactive samples were counted in a liquid scintillation counter (Packard Tri Carb 314 EX).

RESULTS

We have previously reported the effectiveness of pea broth as a sporulating medium (Pheil, Lee, and Ordal, Bacteriol. Proc., p. 17, 1966). Since peas appeared to contain the essential factors required for sporulation, we determined the carbohydrate composition of the peas used. A 20-g amount of Alaskan peas was macerated in a Waring Blendor with 100 ml of water. The macerates were centrifuged, and the supernatant fluids were used for the tests. The glucose content, as determined by the Glucostat method, was less than 0.02 mg/ml, which is the limit of the sensitivity of the test; no glucose spots were present on the paper chromatograms. However, the paper chromatograms showed major spots for arabinose, sucrose, mannose, and fructose. Minor spots were representative of maltose, xylose, and two very slow-moving spots which were not identified. This discovery of large amounts of arabinose and the lack of glucose in pea extract led to the examination of effects of various carbohydrates in the basal medium on sporulation. The results are presented in Table 1. The only carbohydrates which stimulated sporulation were L-arabinose

TABLE 2. *Effect of various concentrations of peptone and yeast extract on the sporulation of Clostridium thermosaccharolyticum 3814*[a]

Nitrogen source	Concn of yeast extract (%)	Final *p*H	Sporulation[b]
0.5% peptone.....	0.5	5.50	43
1.0% peptone.....	0.5	5.25	21
1.0% peptone.....	0.1	6.71	No growth
1.0% peptone.....	0.05	6.76	No growth
0.5% Trypticase[c]..	0.5	5.20	16
1.0% Trypticase...	0.5	6.38	1
1.0% Trypticase...	0.1	6.70	No growth

[a] L-Arabinose (0.5%) was used as the carbon source; the cultures were incubated at 58 C for 23 hr. Each medium contained Gc minerals.

[b] Per cent sporulation was determined by Petroff-Hausser counts.

[c] One ppm of thiamine was added to these samples.

and L-xylose. Glucose and other hexoses, as well as the D form of the pentoses, appeared to repress sporulation. Similar results were also observed with the strains TA-37, TA-3, and TA-4. Although Murrell (12) reported the inhibitory effect of sporulation to be due to the lowering of the *p*H to an unfavorable level, our effort of continuous adjustment of the *p*H to 5.5 ± 0.2 up to 20 hr of incubation with NaOH or by buffering the medium with phosphate or tris(hydroxymethyl)-aminomethane (Tris) buffer did not alter the degree of sporulation of the organism in either L-arabinose or glucose medium.

To further characterize the requirements for sporulation, the effect of the nitrogen source and vitamin concentration was evaluated with L-arabinose as the carbohydrate. The results (Table 2) indicate that maximal sporulation occurred with the combination of 0.5% peptone and 0.5% yeast extract. When the concentration of yeast extract was reduced to 0.1% or lower, no growth resulted. This was presumably due to the lack of essential nutrilites. Increasing the concentration of peptone or replacing it with Trypticase (plus 1 ppm of thiamine) resulted in reduced spore yields. A medium in which the yeast extract was replaced with the vitamin mixture of Mitchell and Clark (11) failed to support growth of the organism. Likewise, the basal medium without carbohydrate (arabinose) did not support growth.

The morphological appearance of the cells in the carbohydrate medium supporting sporulation was distinctly different from that of the cells in the medium in which sporulation was repressed (Fig. 1). Sporulating cells were long (8.9 to 10 μ), thin (0.35 μ), and granular, whereas the nonsporulating cells were shorter (2.0 to 3.5 μ), thicker (0.5 μ), and nongranular.

The changes in *p*H and acetate concentration during growth and sporulation of *C. thermosaccharolyticum* 3814 in Gc broth are presented in Fig. 2. The apparent optimal *p*H for sporulation for all our strains of the "thermophilic anaerobes" was in the range of 5.0 to 5.5. The optimal sporulation time was 18 to 24 hr. This is similar to that reported for the sporulation of *C. butyricum* (13). After prolonged incubation (10 to 14 days) the degree of spore formation was reduced to less than 5%. Thus, a short and critical incubation appears especially characteristic of the predominantly saccharolytic species of the clostridia.

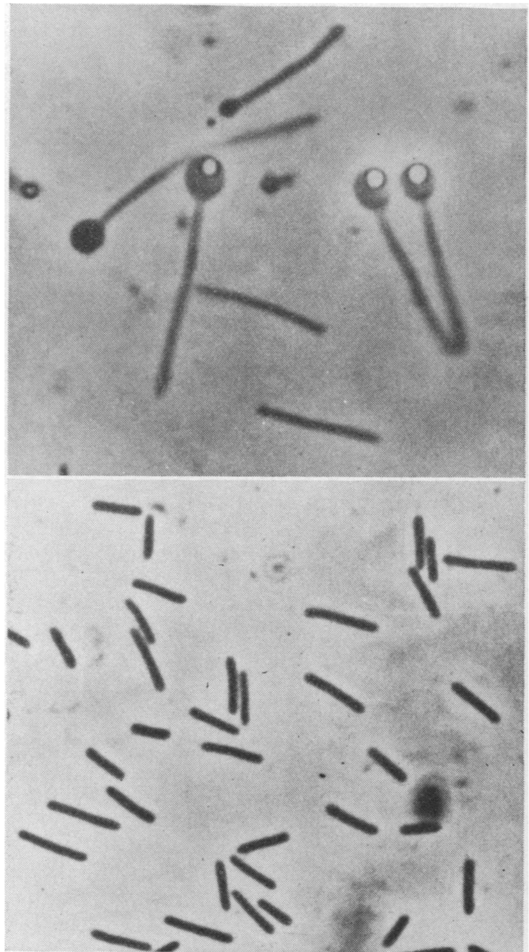

FIG. 1. *Morphology of cells of Clostridium thermosaccharolyticum 3814. The cells were incubated at 58 C for 24 hr. Top: sporulating cells in basal medium plus 0.5% L-arabinose. Bottom: nonsporulating cells in basal medium plus 0.5% glucose.*

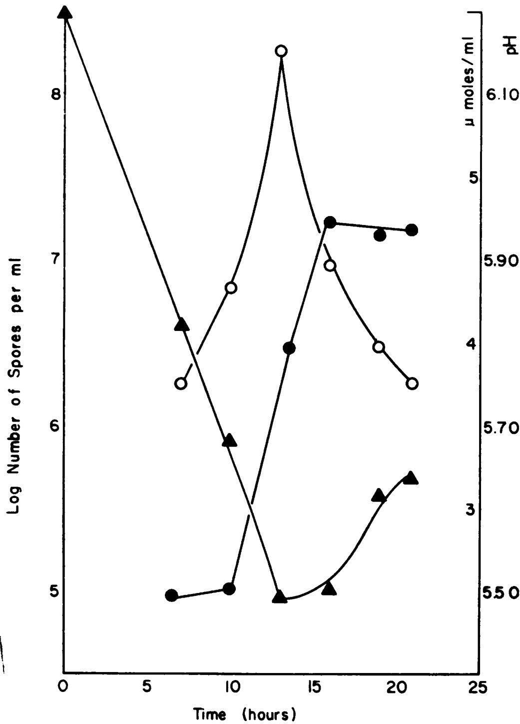

FIG. 2. *Changes in pH and acetate concentration during sporulation of Clostridium thermosaccharolyticum 3814. The cells were grown at 58 C in Gc broth. Symbols:* ○, *acetate;* ●, *spores;* ▲, *pH.*

During sporulation, a rise in *p*H and reduction in acetate concentration was observed; similar findings have also been observed with *C. botulinum* (4) and with many of the bacilli. These results indicate that the L-arabinose is dissimilated to organic acid intermediates during growth and these are utilized during sporogenesis. The reduction of acetate in the medium during sporulation suggests that it may be the source of energy during sporulation or the precursor of important cellular constituent(s) of the spore. Although butyric acid was routinely observed in the culture supernatant fluid, no other short-chain acids (less than 6 carbons) were detectable.

Since acetate appears to have a definite role in sporulation, the incorporation of acetate into the cellular constituents was measured by use of acetate-*2-^{14}C*. The cells were grown in a 125-ml Warburg flask containing Gc broth with hyamine hydroxide in the center well. When the cells reached the early transition stage (long granular cells with some terminal swelling), the labeled acetate was added (1.25 μc/ml).

When maximal sporulation occurred, approximately 20% of the acetate-*2-^{14}C* was incorporated in the acid-hydrolyzed, ether-extractable fraction. As these spores have a fatty acid content of 15 to 16% (15), this would suggest that acetate is an important precursor in their synthesis. The cold trichloroacetic acid-soluble and the hot trichloroacetic acid-soluble fractions contained only 1.3 and 2.0% of the label, respectively. The majority of the remaining label was located in the residue fraction, which contained much of the spore protein.

The average diameter of these spherical spores was 0.83 μ, and the estimated volume (calculated as a sphere) was 0.29 μ^3. The amount of phase-dark material surrounding each spore was variable, but its composition was not determined. As this material took up Sudan Black stain, it was assumed to be high in lipid constituents.

The agreement between the direct microscopic counts (DMC) and viable counts by the most probable number (MPN) technique was, in most cases, good (Table 3). However, the tube counting invariably gave a reduced count for all the suspensions counted. The large amounts of acid and gas produced by this organism during outgrowth, coupled with the wide variation in spore germination time, probably inhibited or killed the slower outgrowing cells, and hence did not produce visible colonies.

Data on the chemical composition of spores produced in two different media are presented in Table 4. Comparable amounts of calcium and phosphorus were present in the two spore suspensions. The magnesium content was significant, whereas those of manganese and iron were comparable to that of some aerobic spores. The percentage of calcium and DPA, and the molar ratio of Ca-DPA, were comparable to those reported for other clostridial spores (4, 20).

TABLE 3. *Enumeration of various spore suspensions*

Spore suspension	Spores per ml (× 10⁹)		
	DMC[a]	MPN[b]	Tube[b]
TA 37	1.04	1.01	0.47
TA 37	1.41	1.33	0.76
3814	0.90	0.62	0.15
3814	1.05	0.93	0.40

[a] The direct microscopic counts (DMC) were determined with a Petroff-Hausser counter.

[b] The spores were heat-activated for 10 min at 100 C; the most probable number (MPN) was determined by the procedure of Halvorson and Ziegler (6) with pea broth. The tube counts were performed with a modified pea extract-agar in screw-cap test tubes (16 by 150 mm).

TABLE 4. *Chemical composition of spores of Clostridium thermosaccharolyticum 3814*

Component	Media for spore production	
	L-Arabinose[a]	Pea broth[b]
Ca	2.55	2.62
P	2.60	2.40
Fe	—[c]	0.13
Mn	—	0.09
Mg	—	1.10
DPA	7.25	—
Lipid	16.3	—
Molar ratio: Ca–DPA	1.47	—

[a] Percentage of dry weight, determined by spectrometric and titration analysis on spores produced in the L-arabinose medium.

[b] Percentage of dry weight, determined by a Jarrell-Ash Emission Spectrograph on spores produced in the modified pea broth.

[c] Not determined.

DISCUSSION

The evidence presented establishes a somewhat uniform picture of the effect of the carbon source on the sporulation of "thermophilic anaerobes." Preliminary evidence is given on biochemical changes during sporulation and the chemical nature of the resulting spores. The sporulation process in *C. thermosaccharolyticum* 3814 was not brought into optimal synchrony, since a vegetative-cell inoculum was used. However, the degree of sporulation was sufficient to correlate functional changes with morphological changes. Sporulation was only observed when the pentoses were utilized as a carbon source, whereas a repression of sporulation was noted with the hexoses and D-pentoses. The reasons for the specificity of the effect of the L-pentoses on clostridial sporulation has not been previously reported. However, Majunder and Padma (10) observed a sporogenic effect of D-xylose in bacilli, but no stimulation with arabinose was noted. The probability of sporulation during growth was also dependent on the nature and concentration of the nitrogen sources. Although the peptones represent complex nitrogen sources, it was interesting to note that peptone exhibited a greater stimulatory role than did Trypticase. It has been suggested by Schaffer et al. (18) that only the enzymes which participate exclusively in carbohydrate metabolism have their synthesis repressed by ternary catabolites, which are formed whether or not a utilizable nitrogen source is present.

The optimal *p*H of a medium specified for sporulation is confusing, since the initial *p*H, the *p*H during growth, and the final *p*H after growth and sporulation are seldom the same. The optimal *p*H for our strains after growth and sporulation was *p*H 5.0 to 5.5. This substantiates that reported for other actively saccharolytic clostridia (1, 2). However, Stumbo (19) reported that the optimal *p*H for sporulation of *C. thermosaccharolyticum* was *p*H 7.0.

The intermediate stages in the development of mature spores from the granular vegetative cells were not defined, but it was possible to correlate the presence of increasing number of spores with a rise in *p*H and a net decrease in the acetate concentration of the culture medium. The decrease in acetate concentration during sporulation is similar to the observations of Day and Costilow (4) with *C. botulinum* type A and with the bacilli (7). The *p*H rise during sporulation also suggests a decrease in the content of free acids or an increased production of ammonia or basic amino compounds, or both.

The incorporation of approximately 20% of the labeled acetate into the firmly bound lipid fraction of spores indicates that some of the acetate was used as a precursor for lipid synthesis. The high lipid content of spores of *C. thermosaccharolyticum* 3814 (15) substantiates this relationship.

The sporulating cells of *C. thermosaccharolyticum* 3814 exhibit a unique morphology. The long, thin, granulated cells in which spores are formed are in direct contrast to the bacilli and to some clostridia in which the vegetative cells shorten and thicken during sporulation. Generally, it is difficult to compare the cell morphology during the sporulation of different genera or species of the same genera. However, the variations in cell morphology as a function of the carbon source present a unique system for studying the factors influencing the cellular changes during sporulation.

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