An Appraisal of *Caryophanon latum*

By P. J. PROVOST* AND R. N. DOETSCH

*Departments of Microbiology and Dairy Science, University of Maryland,
College Park, Maryland, U.S.A.*

(Received 25 September 1961)

SUMMARY

Cultures of the giant bacterium *Caryophanon latum* Peshkoff, among them our own isolates from cow dung, were studied. A method for isolation is described. A fluid medium containing acid-hydrolysed casein, thiamine, biotin, sodium acetate, sodium butyrate and dipotassium phosphate, at pH 7·8, was devised which, without added colloids, allowed luxuriant growth of *C. latum* when sufficient aeration was provided. *C. latum* has been found to utilize acetic, butyric and β -hydroxybutyric acids. A variety of other carbon sources did not appear to be readily utilized by *C. latum*. The organism produced a carotenoid-like yellow pigment, the synthesis of which was inhibited by diphenylamine. A large amount of poly- β -hydroxybutyric acid (PHB) was synthesized by *C. latum* growing in the fluid medium described above. Much of the PHB was liberated as free granules. *C. latum* was found to be Gram-positive, in contrast to previously published reports.

INTRODUCTION

The giant bacterium *Caryophanon latum* is 3μ wide $\times$ 15μ long, as an average. Although a number of papers concerning its cytology have appeared (Peshkoff, 1940, 1946; Pringsheim & Robinow, 1947; DeLamater, 1952; Bisset, 1953; Sall & Mudd, 1955; Tomcsik & Grace, 1955; Murray, 1957), there is little information about the ecology, nutrition and basic physiology. *C. latum* was first described by Peshkoff (1940); it was isolated from cow dung and was cultivated on manure extract agar but not on 'ordinary' media. Pringsheim & Robinow (1947) re-isolated *C. latum* from cow dung, presented a definitive description of its structure, and contributed materially toward an understanding of its ecology and nutrition. The organism did not appear to be an inhabitant of the bovine intestine. It could be grown on an agar medium (PYA) containing 0·5% (w/v) Bacto peptone, 0·5% (w/v) Bacto-yeast extract, and 0·01% (w/v) sodium acetate, at pH 7·4 to 7·6, but grew poorly, if at all, in fluid media unless these were supplemented with small amounts of agar. The first report of an effort to grow *C. latum* with defined nutrients was published by May & Gershenfeld (1955). These workers used a medium containing soluble sodium silicate, hydrolysed cellulose, salts, asparagine and pantothenic acid. Weeks & Kelley (1958) were not able to grow *C. latum* well in fluid media (PYA less agar) even with the incorporation of various types of particulate matter.

* Present address: Department of Microbiology, School of Medicine, University of Maryland, Baltimore 1, Maryland, U.S.A.

Somewhat better growth was obtained in cow-dung extract fluid medium. The present work reappraises some parts of previous studies and supplies new information about the nutrition and physiology of *Caryophanon latum*.

METHODS

Isolation of Caryophanon latum cultures. Intensive efforts to isolate *C. latum* from nature were made only with cow dung as source material. Relatively fresh (from a few hours to one day old) cow dung samples were collected from pastures, barns and pens. These samples upon return to the laboratory were incubated for one day at room temperature (about 20°) in glass bottles with loose screw tops. Wet mount preparations were then examined microscopically ($\times 440$) for typical *C. latum* trichomes. Most samples thus examined did not show *C. latum*. However, some positive samples were found; isolations of *C. latum* were made from dung samples found in close association with hay and straw, and from a pasture dung sample.

For isolation, a positive sample was divided into two parts; one part was autoclaved (121°, 15 min.) and inoculated with several loopsful of the remaining portion. After incubation for 24 hr. at 25°, the inoculated dung teemed with *Caryophanon latum* trichomes; a cultural enrichment was thus obtained. *C. latum* appeared to grow somewhat better in cow dung than most other bacteria present in the inoculum. The enriched sample was suspended in water (about 2 g. in 10 ml.), and the suspension forced through several layers of gauze to remove large particles. The filtrate was centrifuged at 50 g for 8 min., the sediment resuspended in water and recentrifuged as many as 11 times, with discard of the supernatant fluid each time. A mechanical enrichment of the relative numbers of *C. latum* was achieved because the large trichomes sedimented with this low-speed processing. The resulting sediment was streaked on plates of PYA medium (Pringsheim & Robinow, 1947). After 24 hr. at 25°, the plates were examined microscopically for minute colonies of the granular greenish appearance typical of *C. latum*; twelve cultures were isolated and purified by continued streaking.

Cultures were obtained from the American Type Culture Collection (Washington, D.C., U.S.A.; ATCC 12871 and ATCC 13872), Dr E. G. Pringsheim (Göttingen University, Germany), and Dr T. Gibson (University of Edinburgh); these cultures, with our own isolates, formed the collection on which the work reported here was based. The cultures were maintained on PYA slopes at 4° and transferred at monthly intervals.

Standard procedures. All fluid cultures were incubated at 25° on a rotary shaker (about 200 rev./min.) to provide vigorous aeration. For tube cultures, racks were constructed which held up to 50 tubes (16 $\times$ 150 mm.) at an angle of about 40° from horizontal and which could be clamped to the shaker platform.

Inocula were prepared by suspending organisms from PYA slopes grown for 24 hr. at 25°, in 5 ml. water or fluid medium. Four ml. were removed and the organisms washed (3 times in vitamin requirement studies, once or twice in most other experiments), with centrifugation periods of 5 min. at 500 g. The washed organisms were finally resuspended in a volume of 4 ml. (optical density at 500 m μ about 0.2). Inocula were added to fluid media at 1 % (v/v) of the medium volume.

Turbidimetric measurements were made with a Bausch & Lomb ('Spectronic 20' colorimeter, at 625 m μ in coloured media and at 500 m μ in colourless media. Optically matched 16 $\times$ 150 mm. Pyrex culture tubes were used in growth experiments.

Dry-weight determinations were made with 3 ml. samples in tared glass cups held at 90–100° for 24 hr. and cooled over CaCl₂ before weighing.

Sterilization of fluid media was usually done with 0.45 μ pore diameter membrane filters (Millipore Filter Corp., Bedford, Mass., U.S.A.).

The following staining procedures were used: the Hucker modification of the Gram stain and the Burdon lipid stain (*Manual of Microbiological Methods*, 1957), and a negative stain with an aqueous 7% (w/v) filtered suspension of nigrosin.

Nutritional studies. A fluid medium was developed containing: 1% (w/v) 'salt-free' acid-hydrolysed casein (Hy-Case SF, Sheffield Chem. Co., Norwich, N.Y., U.S.A.); 0.5% (w/v) sodium acetate, anhydrous; 0.5% (w/v) sodium butyrate; 0.1% (w/v) K₂HPO₄; pH 7.8–7.9. When supplemented with a mixture of B-vitamins, this medium supported good growth of *Caryophanon latum*. Aqueous solutions of the B-vitamins were prepared at $\times 100$ the final desired medium concentrations (see Table 1 for final concentrations) and added to or individually deleted from the above basal medium. Growth responses were measured turbidimetrically. PYA medium, without added agar, and the defined medium of May & Gershenfeld (1955) were also examined for ability to support the growth of *C. latum*.

Utilization of carbon and energy sources. The ability of *Caryophanon latum* to utilize a variety of carbon compounds was tested by measuring growth responses when these compounds were added to a basal medium incapable of supporting growth unless supplemented with a utilizable carbon and energy source. The basal medium contained: 1% (w/v) Hy-Case SF; 0.1% (w/v) K₂HPO₄; thiamine HCl 0.2 mg./l.; biotin 0.04 mg./l.; pH 7.8–7.9. This basal medium is referred to as CHB.

Sodium acetate and sodium butyrate, sterilized in aqueous solution by membrane filtration, were tested at various concentrations. Carbohydrates, sterilized with ethylene oxide, were added to CHB medium at 0.5% (w/v). The C₅–C₉ saturated fatty acids (as sodium salts, sterilized by autoclaving) were tested at final concentrations of 0.2% (w/v). Tricarboxylic acid cycle and related compounds (formate, pyruvate, propionate, oxaloacetate, malate, α -ketoglutarate, fumarate, succinate; as sodium salts, sterilized both by membrane filtration or autoclaving) were added to CHB medium at 0.02M to detect utilization. In later work, 'sparker' concentrations of acetate and butyrate (0.025% w/v) were incorporated into CHB medium for retesting the ability of *Caryophanon latum* strain 1.8 to utilize glucose, pyruvate, caproate and α -ketoglutarate.

To detect oxygen uptake on the sodium salts of the C₂–C₉ saturated fatty acids by resting suspensions of *Caryophanon latum*, conventional Warburg equipment was used. Each flask contained 3.1 ml. total fluid, consisting of: 2 μ mole of fatty acid (Na salt) in 0.1 ml. water, 1 ml. 0.067M-phosphate buffer (pH 7.5), 1 ml. of washed suspension of organism (equivalent to about 1.3 mg. dry wt.); 0.9 ml. water; 0.1 ml. of 20% (w/v) KOH in the centre well containing a piece of fluted filter paper. The temperature of the water bath was maintained at 24.9°. The resting organisms were used as soon as prepared, with no starvation period.

Pigment studies. Four-day cultures in CHM medium (100 ml.) were harvested

and washed twice in 0.85% (w/v) NaCl. A concentrated suspension (2 ml.) was spread on filter paper and dried over CaCl_2 . (Extraction with acetone removed most of the pigment.) The dried organisms were extracted with 50 ml. boiling methanol for 5 min., the extracts filtered (Whatman no. 50 paper) and their optical density values from 230 to 700 $m\mu$ at 10 $m\mu$ intervals determined in a Beckman DU spectrophotometer (1 cm. silica cells, 3 ml. volume) with a methanol blank. Diphenylamine was incorporated into CHM medium at 0.02–0.10 mg./ml. medium to determine whether inhibition of pigment synthesis would occur. Methanol extracts were prepared and examined, as described above, from the amine-containing cultures.

Poly- β -hydroxybutyric acid (PHB) studies. Washed CHM culture harvests were dried either *in vacuo* over CaCl_2 or by lyophilization. The dried material was first extracted for several hours in a Soxhlet apparatus with anhydrous di-ethyl ether which removed yellow cell pigment and much lipid material, and then extracted to exhaustion with chloroform; the latter removed the PHB. Dry weights of chloroform extracts were determined by boiling away the chloroform from measured samples. Samples of crude extracts were tested for melting point and solubilities (see Williamson & Wilkinson, 1958) and for absorption spectrum when dissolved in hot (100°) concentrated H_2SO_4 (Slepecky & Law, 1960). An assay for the PHB in the extracts, similar to that of Law & Slepecky (1961), but with the use of a crotonic acid standard curve, was performed. Crotonic acid used as the standard was purified by recrystallization from water three times.

The utilization of sodium- β -hydroxybutyrate by *Caryophanon latum* was determined by incorporating this compound (0.02M and 0.5%, w/v, membrane-filtered) into CHB medium and measuring growth responses turbidimetrically.

General studies. A solid medium containing acid-hydrolysed casein, yeast extract and sodium acetate (CYA, see Results) was used in determining the biochemical capacities of *Caryophanon latum* as concerns the production of catalase, reduction of nitrate (0.1%, w/v, KNO_3 added), production of indole (0.1%, w/v, DL-tryptophan added), and liquefaction of gelatin (agar replaced with 12% w/v, gelatin). These tests were done as outlined in the *Manual of Microbiological Methods* (1957). Studies of the relation to oxygen (in deep shake tubes of CYA medium) and to culture survival on PYA slopes were also done.

The effect of lysozyme was tested on organisms suspended in an aqueous solution containing 7.5% (w/v) polyethylene glycol, 0.01M-NaCl, 0.005M- MgSO_4 , and lysozyme 375 $\mu\text{g./ml.}$ at pH 7.6.

The effects of antibiotics and sulfa drugs ('Sensi-Discs', Baltimore Biological Laboratory, Inc., Baltimore, Md., U.S.A.) were tested on plates of PYA medium. The effects of crystal violet (1/100,000) and sodium azide (0.03%, w/v) were determined by incorporation of these compounds into PYA medium.

RESULTS

Growth of Caryophanon latum in fluid media

Table 1 shows typical results obtained in the vitamin-omission studies. Lessened growth of both *Caryophanon latum* cultures tested occurred only upon the omission of thiamine HCl and biotin. Thiamine HCl was required for growth. The requirement

for biotin was not so clear-cut; some growth occurred when it was omitted, but this was accompanied by heavy clumping. Further testing, with thiamine HCl as the only vitamin added to the basal medium, showed (lower part of Table 1) that it alone could not satisfy the vitamin requirements of *C. latum*. The addition of biotin allowed luxuriant growth of both cultures, thus indicating a requirement for both thiamine HCl and biotin.

Table 1. *Vitamin requirements of Caryophanon latum*

Growth responses are expressed as optical density values at 625 m μ . The basal medium contained 1% (w/v) Hy-Case SF, 0.5% (w/v) each of sodium acetate and sodium butyrate, 0.1% (w/v) K₂HPO₄; pH 7.8. Vitamins and concentrations were (mg./l.): *p*-aminobenzoic acid, 0.2; biotin, 0.04; folic acid, 0.04; cyanocobalamine, 0.015; Ca-D-pantothenate, 0.04; nicotinic acid, 0.2; pyridoxal HCl, 1.0; riboflavin, 0.2; thiamine HCl, 0.2.

	Culture			
	Isolate 1.8		ATCC 12871	
	48 hr.	96 hr.	48 hr.	96 hr.
Vitamins omitted				
All	0.12	0.08	0.01	0.02
None	1.03	1.82	1.15	1.22
Thiamine HCl	0.14	0.12	0.01	0.02
Biotin	0.48	0.50	0.80	0.88

	Culture			
	Isolate 1.8		ATCC 12871	
	48 hr.	120 hr.	48 hr.	120 hr.
Vitamins added				
None	0.07	0.05	0.01	0.03
Thiamine HCl	0.06	0.08	0.02	0.06
Thiamine HCl + biotin	1.18	1.96	0.01	1.66

With the addition of these two growth factors, a fluid medium (CHM) for the growth of *Caryophanon latum* was developed. It consisted of (in g./l.) Hy-Case, 10.0; sodium acetate, anhydrous, 5.0; sodium butyrate, 5.0; K₂HPO₄, 1.0; and (in mg./l.) thiamine HCl, 0.20; and biotin 0.04; pH 7.8–7.9. This medium was usually sterilized by filtration through an 0.45 μ pore diameter membrane filter, but it could be autoclaved (121°, 10 min.). Removal of acetate and butyrate from CHM medium resulted in a basal medium (CHB) which did not allow extensive growth of *C. latum*; CHB medium was used in the carbon and energy source utilization studies (see above).

Large crops of organism (2.0–2.5 g. dry wt./l. in 72 hr.) were produced in CHM medium with adequate aeration. Typical growth responses, of representative *Caryophanon latum* cultures in CHM medium, with and without aeration, are shown in Table 2. All cultures, except isolate 2.7, grew in the medium on continued transfer. Culture ATCC 12871 showed a rather long lag period which was characteristic of this culture when grown in CHM medium. The lag seemed to be overcome to some extent on continued transfer in CHM medium (Table 2). The addition of 0.1% (w/v) Bacto yeast extract to CHM medium allowed the growth of culture isolate 2.7 and overcame the lag period of culture ATCC 12871. The addition of salts (MgSO₄·7H₂O, 0.25 mg./ml.; MnSO₄·H₂O, 0.01 mg./ml.; FeSO₄·7H₂O, 0.01 mg./ml.)

allowed the production of a more intense yellow pigmentation. The acid-hydrolysed casein in CHM medium could not be replaced with 0.5% (w/v) $(\text{NH}_4)_2\text{SO}_4$; no attempts were made to replace it with known amino acids and peptides.

Without vigorous aeration, none of the *Caryophanon latum* cultures grew well, even in CHM medium. Table 2 shows typical growth responses of still (non-shaken) cultures (5 ml. amounts in 16 × 150 mm. culture tubes).

Table 2. *Growth of representative Caryophanon latum cultures upon serial transfer with vigorous aeration in CHM medium and growth in CHM medium without aeration*

Figures in parentheses indicate day on which optical density shown was attained and also day when one loopful of culture was transferred to next set.

Culture	Source	Aerated			Non-aerated	
		Set no. 1	Set no. 2	Set no. 3	72 hr.	144 hr.
		Optical density at 625 m μ				
P	E. G. Pringsheim	1.82 (2)	1.70 (2)	1.92 (2)	0.08	0.08
PG	T. Gibson	1.52 (3)	1.70 (3)	1.52 (2)	0.03	0.075
12871	ATCC	1.26 (4)	2.0 (2)	2.0 (3)	0.02	0.18
13872	ATCC	1.70 (2)	1.70 (2)	1.52 (2)	0.02	0.06
1.8	Present isolate	1.70 (1)	1.70 (2)	1.70 (1)	0.11	0.19
1.2	Present isolate	1.70 (1)	1.52 (1)	1.70 (1)	0.14	0.20
2.7	Present isolate	0.02	—	—	0.02	0.03
2.9	Present isolate	1.70 (3)	1.70 (2)	1.60 (2)	0.02	0.085
3.1	Present isolate	1.52 (2)	1.52 (3)	1.52 (3)	0.05	0.12

A solid medium (CYA) was developed for the routine culture of *Caryophanon latum*. This medium consisted of (in g./l.) Hy-Case SF, 10.0; Bacto yeast extract, 5.0; sodium acetate, anhydrous, 5.0; and agar, 15.0; pH 7.8–7.9.

Considerably greater crops of organism were produced on this medium than on the PYA solid medium of Pringsheim & Robinow (1947). The higher concentration of acetate (0.5%, w/v) in the CYA medium (PYA contains only 0.01%, w/v) was probably responsible for the enhanced growth.

PYA medium, in a fluid form without added agar, did not yield large crops even with vigorous aeration. Maximum optical density values at 625 m μ produced in this medium were in the range of 0.30–0.40 with isolate 1.8 and ATCC 12871. Omission of the acetate 0.01%, (w/v) did not markedly affect the amount of growth, suggesting that there was too little acetate in PYA medium to have much effect as an energy source.

A study of the effects of the nutrients in the defined medium of May & Gershenfeld (1955) revealed the following. Asparagine (0.5%, w/v) could not replace the Hy-Case SF of CHM medium. Asparagine + pantothenate (0.04 mg./l.) + riboflavin (0.2 mg./l.) + salts (mg./ml.): $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; KCl, 0.25; $\text{Na}_2\text{SO}_4 \cdot \text{H}_2\text{O}$, 0.01; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 could not replace the Hy-Case SF. Glucose (0.5%, w/v) could not substitute for the acetate and butyrate in CHM medium. It would appear that the defined medium of May & Gershenfeld was not able to yield large crops of *Caryophanon latum*. Sodium silicate was not tested since the present work showed that *C. latum* did not require colloidal material for growth in fluid media.

Carbon and energy sources for Caryophanon latum

Manometric studies (Table 3) showed that *Caryophanon latum* oxidized acetic and butyric acids, but not other saturated fatty acids. *C. latum* isolate 1.8 resting organisms, from growth in CHM medium supplemented with 0.3% (w/v) each of sodium caproate and heptanoate, showed no enhanced ability to oxidize the C₅–C₉ acids.

Table 3. *Oxidation of fatty acids by resting suspensions of Caryophanon latum isolate 1.8*

Flasks contained equiv. 1.3 mg. dry wt. organisms harvested at 24 hr. from CHM medium, washed three times in 0.85% (w/v) NaCl in water. The fatty acids were used as sodium salts.

Substrate (2 μ mole)	μ l. O ₂ (5 hr.)	Q_{O_2} (average μ l. O ₂ /mg. dry wt./hr.)
None	67.5	9.4
Acetate (C ₂)	138.0	19.3
Propionate (C ₃)	60.5	8.5
Butyrate (C ₄)	136.0	19.1
Valerate (C ₅)	74.0	10.3
Caproate (C ₆)	81.5	11.5
Heptanoate (C ₇)	81.0	11.3
Octanoate (C ₈)	74.5	10.4
Pelargonate (C ₉)	67.0	9.4

Table 4. *Stimulation of growth of Caryophanon latum by acetate and butyrate*

Figures in parentheses indicate day on which optical density shown was reached.

Addition to CHB medium*	Organism	
	Isolate 1.8	ATCC 12871
	Max. turbidity (optical density at 625 m μ)	
None	0.005	0.005
0.05 M-sodium acetate	0.62 (2)	1.21 (4)
0.05 M-sodium butyrate	1.70 (6)	2.0 (5)

* See Results for composition of CHB.

Of a variety of fatty acids, tricarboxylic acid cycle acids, carbohydrates and related compounds tested, only acetate and butyrate allowed substantial growth of *Caryophanon latum* when added to CHB medium. Table 4 shows typical responses of *C. latum* to acetate and butyrate. The concentrations (0.05 M) are far in excess of the minimum amounts of these acids necessary for growth stimulation; namely, about 0.01 M. A stimulatory effect on butyrate utilization in the presence of acetate (Table 5) in isolate 1.8 was observed.

No substantial growth stimulation of isolate 1.8 or ATCC 12871 was provided by any of the following, added singly to CHB medium: C₅–C₉ saturated fatty acids at 0.2% (w/v) (some growth on the C₅ and C₈ acids with both cultures after 8–11-day lag periods); glycerol, arabinose, glucose, galactose, maltose, cellobiose, soluble starch at 0.5% (w/v) each; formate, pyruvate, propionate, oxaloacetate, malate, α -ketoglutarate, fumarate, succinate, all as sodium salts at 0.02 M each (slight

turbidities, OD = 0.02–0.04 at 625 m μ , were produced on malate, α -ketoglutarate, fumarate). The addition of small amounts of acetate and butyrate (0.025 %, w/v, each) to CHB medium and the subsequent testing of isolate 1.8 for growth on glucose, pyruvate, caproate, or α -ketoglutarate (concentrations as above) showed apparent utilization of caproate and α -ketoglutarate, but not glucose and pyruvate; this was deduced from the greater turbidities in the former than were produced in the low acetate + butyrate medium without supplementation.

Table 5. *Stimulation of butyrate utilization by acetate with Caryophanon latum* 1.8

Addition to CHB	Optical density at 625 m μ		
	24 hr.	48 hr.	144 hr.
None	0.01	0.01	0.01
0.5 % (w/v) sodium acetate	0.37	0.80	0.42
0.5 % (w/v) sodium butyrate	0.02	0.02	1.73
0.5 % (w/v) of both acetate and butyrate	1.24	1.50	1.87

Caryophanon latum pigment

A yellow pigment was prominent with all organisms of our collection of *Caryophanon latum* when large crops were obtained. Diphenylamine inhibited the production of the pigment, but not growth of isolate 1.8, at 0.02, 0.04 and 0.06 mg./ml. in CHM medium. Growth of culture ATCC 12871 was inhibited by lower concentrations of diphenylamine (0.04 mg./ml.). Methanol extracts of 4-day CHM cultures of *C. latum* isolate 1.8, grown with and without 0.02 or 0.04 mg. diphenylamine/ml., showed an absorption peak at 430 m μ in the non-inhibited cultures but no strong absorption in this range in the inhibited cultures. From the absorption peak of the *C. latum* pigment and previous work (Ellinghausen & Pelczar, 1957) which indicated diphenylamine to be an inhibitor of carotenoid pigment synthesis, it seems likely that the *C. latum* pigment is carotenoid.

Poly- β -hydroxybutyric acid (PHB) in Caryophanon latum

Caryophanon latum cultures grown in CHM medium presented a cloudy opalescent supernatant portion on standing for several hours after removal from the shaker. Staining with basic dyes revealed no particulate matter in these supernatant fluids, but negative staining with nigrosin showed small spherical refractile granules (Pl. 1, fig. 1). These free granules did not stain well with the Burdon lipid material stain (Pl. 1, fig. 2). Intact *C. latum* trichomes settled to the bottom in still cultures, and thus were not responsible for the supernatant turbidity. The supernatant portion (mostly granules) of settled cultures could account for a large percentage (Table 6) of the particulate dry weight of CHM cultures. The free granule material (supernatant) increased rapidly about one day after the initial rise in culture turbidity. Analysis of chloroform extracts of CHM culture harvests revealed large amounts of a material with the properties of PHB as described by Williamson & Wilkinson (1958) and with a peak absorption at 235 m μ when dissolved in hot (100°) concentrated H₂SO₄ (Slepecky & Law, 1960). Thus, it appeared that the supernatant granules were mainly PHB granules, probably liberated on lysis of the organisms. Table 6 also shows an assay for the PHB content of a 6-day CHM culture.

The percentage PHB is higher than any previously reported; but it should be borne in mind that a considerable degree of lysis had occurred, thus decreasing the particulate dry weight due to organisms. Analysis of a 4-day CHM culture showed a PHB content of about 50 %.

The PHB appeared to be synthesized mainly from butyrate; but organisms grown only with acetate in CHB medium also showed some, though lesser, accumulation of lipid granules. The large synthesis of PHB in CHM medium may have reflected a high C:N ratio in this medium. Increasing the Hy-Case SF content of CHM to 3 % (w/v) did not diminish the production of PHB. When tested manometrically, no utilization of PHB itself was found, but isolate 1.8 and ATCC 13872, the only strains investigated in this regard, did grow in CHB medium supplemented with either 0.25 % or 0.5 % (w/v) sodium- β -hydroxybutyrate.

Table 6. *Data on free lipid granule and poly- β -hydroxybutyric acid (PHB) content of 6-day CHM culture of Caryophanon latum isolate 1.8*

Two 15 ml. portions were removed from the 6-day culture and allowed to settle for about 24 hr. The supernatant granule sample was taken from one; the other was resuspended to provide a sample representative of the whole culture. The clarified medium was prepared by centrifugation at 11,000 g for 15 min.

Culture fraction	g. dry wt./ 3 ml. sample	Percentage of particulate dry weight
Supernatant granules	0.0043	60.5
Suspended culture	0.0071	100.0
Clarified medium	0.0428	—
Total culture crop dry wt.	1.2371 g.	—
Chloroform extract dry wt.	0.94 g.	(76 % of total crop dry wt.)

Poly- β -hydroxybutyrate: 80 % of chloroform extract, 61 % of total crop dry wt.

General properties of Caryophanon latum

In contrast to the reports of previous workers (with the possible exception of Tuffery, 1953), in the present work *Caryophanon latum* was found to be Gram-positive. PYA slope cultures, stained at 13, 15, 19, 24 and 48 hr., were uniformly Gram-positive when the Hucker modification of the Gram stain, or a further modification with the crystal violet concentration decreased from 2 to 0.5 % (w/v) and an increase in decolorization time to 75 sec. were used.

Caryophanon latum produced catalase and did not reduce nitrate to nitrite, produce indole, or liquefy gelatin. CYA shake tubes showed growth of *C. latum* only at the surface, with the exception of culture isolates 1.2 and 1.8 which grew to a depth of about 1 mm. PYA slope cultures grew at 10°, 19°, 25°, and 37°, none grew at 45° and 25° seemed optimal. Cultures of *C. latum* ATCC 12871 survived for 9 weeks (not tested further) on PYA slopes at -20°, 4°, 25° and 37°. *C. latum* cultures survived for 9 months (not tested further) at room temperature (about 20°) on tightly enclosed PYA slopes. Cultures have also survived lyophilization for one year.

Spheroplasts (protoplasts?) were produced with lysozyme from culture ATCC 12871. Continuing work (F. Tyeryar, private communication) has indicated a general susceptibility of *Caryophanon latum* to the action of lysozyme. *C. latum*

cultures were resistant to a variety of sulfa drugs but not antibiotics such as penicillin, bacitracin, tetracycline and others. Crystal violet and sodium azide also were inhibitory.

DISCUSSION

Although isolated to date only from cow dung, *Caryophanon latum* does not appear to be an intestinal inhabitant. The obligate aerobic nature of *C. latum* provides perhaps the most convincing reason why it could not be an inhabitant of the anaerobic intestinal tract. Like Pringsheim & Robinow (1947) and Weeks & Kelley (1958) we were not able to isolate *C. latum* from freshly voided dung, i.e. dung which had not come in contact with soil, hay, grass, dust or insects. It would seem likely that these materials are natural harbourers of *C. latum* and that only by chance does the organism reach the cow dung wherein it rapidly proliferates. The fatty acid content (e.g. acetic and butyric acids) and slightly alkaline pH value, about 7.5, of cow dung are probably major factors responsible for allowing the proliferation of *C. latum*; medium CHM is similar to cow dung in these characters.

A greater oxygen uptake (about 2.5 times) on butyric acid might be expected than on acetic acid. The equal oxygen uptakes obtained in the present work might be explained either of two ways; it might be argued that a larger percentage of butyrate than of acetate was assimilated into storage material. Measurements of O₂ uptake with butyrate (up to 24 hr.) did not exceed that observed with acetate at any time. In addition, O₂ uptake on fatty acids C2 through C4 was linear throughout the experimental period. A second and more interesting possibility is that butyrate might have protected the stored poly- β -hydroxybutyric acid in the cells from being metabolized (compare Doudoroff & Stanier, 1959), while acetate conferred no such protection; thus the endogenous metabolism on butyrate might have been far less than on acetate. The high endogenous activity obtained in the present experiments serves as the only indication that *C. latum* can utilize poly- β -hydroxybutyrate.

This compound has been demonstrated in a variety of bacteria (Lemoigne, 1927; Forsyth, Hayward & Roberts, 1958; Levine & Wolochow, 1959; Doudoroff & Stanier, 1959). The amount of poly- β -hydroxybutyrate shown in *Caryophanon latum* in the present work is somewhat greater than that reported for any other bacterium. However, it should be considered that the amount of accumulation of a storage product such as poly- β -hydroxybutyrate is a reflexion of the chemical environment of the organism, as discussed by Herbert (1961). Our fluid medium (CHM) has a high carbon:nitrogen ratio, providing conditions under which heavy accumulation of carbon storage products (such as poly- β -hydroxybutyrate) would be expected.

The nutritional and physiological characters of *Caryophanon latum* revealed thus far do not indicate that this giant bacterium differs in any basic way from other heterotrophic eubacteria. The present work has not been concerned with the cytology of *C. latum* but, considering the structure as elucidated by Pringsheim & Robinow (1947), it seems to us that this structure differs from that observed in the multicellular forms of bacilli only in regard to the frequency of septation, rather in the nature or order of formation. If this be so, it seems that a rather convincing argument could be presented for classifying *C. latum* among the eubacteria rather than in a separate order.

This work was supported in part by a summer fellowship awarded by the National Science Foundation, and by a training grant from the Department of Health, Education and Welfare (2G-615-MTC). This paper is Scientific Article No. A-938 contribution no. 3296 of the Maryland Agricultural Experiment Station.

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EXPLANATION OF PLATE 1

Fig. 1. Nigrosin preparation of the sediment (24 hr. settling) in a 6-day CHM culture of *Caryophanon latum* isolate 1.8. Note the free poly- β -hydroxybutyrate granules and the disintegrating and misshapen trichomes. $\times 1330$.

Fig. 2. Burdon lipid stain of a 24 hr. CHM culture of *Caryophanon latum* isolate 1.8. The dark areas represent lipid. $\times 1330$.