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CLOSTRIDIUM LACTO-ACETOPHILUM NOV. SPEC. AND THE ROLE OF ACETIC ACID IN THE BUTYRIC ACID FERMENTATION OF LACTATE

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Although the fermentation of lactate by butyric acid bacteria has been studied by a number of eminent bacteriologists (Schattenfroh and Grassberger, 1900; Bredemann, 1909; Boekhout and yan Beynum, 1929), including Pasteur (1862) and Beijerinck (1893), it is still a very poorly understood process. A natural butyric acid fermentation of lactate has been reported to occur commonly in low grade silage (van Beynum and Pette, 1936), and, under laboratory conditions, several investigators have easily obtained crude enrichment cultures of butyric acid bacteria using lactate as the main substrate, but nearly all attempts to isolate and propagate these bacteria in pure culture on a lactate medium have failed. In pure culture the bacteria appear to lose their ability to attack lactate, although they can be cultured easily on sugar-containing media. Van Beynum and Pette (1935) finally succeeded in growing these organisms in a lactate medium, but only when an unusually high concentration of yeast autolysate was also provided. Consequently one cannot decide whether the lactate or the yeast autolysate provided the main carbon and energy source. Van Beynum and Pette rightly observe: "There is much uncertainty about the lactate fermentation. As a matter of fact one does not know if it exists as a separate phenomenon, and very little is yet known about the relation between lactate and sugar fermentations."

In the present investigation we have used lactate-decomposing bacteria obtained by the enrichment culture method. By studying their nutritional requirements and chemical activities we have found why butyric acid bacteria cannot be grown in a simple lactate medium and have shown that acetic acid plays an important role in their metabolism.

EXPERIMENTAL RESULTS

Enrichment and isolation of lactate-fermenting bacteria. The investigation was begun by the enrichment and isolation of lactate-fermenting butyric acid bacteria from soil. A medium of the following composition (medium 1) in grams per 100 ml was used: sodium lactate, 1; yeast autolysate, 0.3; $(NH_4)_2SO_4$, 0.05; $MgSO_4 \cdot 7H_2O$, 0.01; K_2HPO_4 , 0.05; $FeSO_4 \cdot 7H_2O$, 0.002; pH 7, made up with tap water. The medium was inoculated with a small quantity of garden soil and incubated anaerobically at 37 C. Within 36 hours the medium became tur-

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bid, considerable gas was given off, and the pH rose to 8.2 to 8.4. After 72 hours a determination of lactic acid by the method of Friedemann and Graeser (1933) showed that the lactate was completely decomposed.

Several soils of different types were used as inocula for enrichment cultures of this type, and in every instance a vigorous fermentation was obtained within 30 to 48 hours. The predominant bacteria were always medium-sized, actively motile rods, a few of which usually contained oval, central, or subterminal spores. In addition, a few small nonsporulating rods and vibrios were always present when the inoculum was not pasteurized. When a pasteurized soil inoculum was used, a more homogeneous but always less vigorous culture was obtained.

In order to eliminate extraneous bacteria as far as possible before attempting the isolation of pure cultures, one or two successive transfers were made in the same enrichment medium. These cultures always developed within 30 to 40 hours, but were notably less vigorous than the original.

Pure cultures were obtained without special difficulty from the enrichment cultures by the shake culture method of Burri (1902). A solid medium of the same composition as the enrichment medium proved to be satisfactory. Oxygen was removed from the culture tubes by the use of a mixture of pyrogallol and potassium carbonate. Within 20 to 30 hours' incubation at 37 C, the agar in the more heavily inoculated tubes was split by gas, but at the higher dilutions a few wellisolated colonies developed without apparent gas formation. After about 24 hours' incubation at 37 C colonies were visible up to the seventh or eighth dilution, but only at lower dilutions was the agar split by gas.

The colonies of the lactate-decomposing bacteria are generally compact, fluffy, dark gray spheres composed of filamentous outgrowths. They are coarsely lobed and rough-edged; they eventually reach a diameter of 1 to 2 mm. The consistency of the colonies is such that the organisms can be easily drawn into a micropipette or transferred with an inoculating needle.

By the repeated use of the shake culture method, nine strains were isolated in pure culture. Each was derived from a different soil.

Morphological characteristics. All nine strains are very similar in appearance (figure 1). The average cell dimensions are 0.8 by 4.6 microns, the variation in width being from 0.7 to 0.9 microns and in length from 3 to 8 microns. Spores develop after about 40 hours' incubation in a favorable medium; they are oval in shape and are usually located subterminally, where they cause a distinct bulge in the cell. The average spore size is 1.1 by 1.5 microns. Young vegetative cells are actively motile by means of 20 or more peritrichous flagella. At this stage they are gram-positive. In old cultures most of the cells become gramnegative. Cells generally occur singly or in pairs, though short chains can be seen. When first isolated, three strains appeared to possess small capsules, but on subsequent cultivation in a variety of media, no capsules could be observed in any of the strains.

Physiological and cultural characteristics. All nine strains are similar in physiological characteristics. They are obligate anaerobes and their development is favored by the addition of a reducing agent such as sodium thioglycolate to the

medium. The following compounds are readily fermented when supplied in a basal medium containing 3 volumes per cent yeast autolysate and the usual salts: glucose, fructose, galactose, mannose, xylose, arabinose, rhamnose, lactose, sucrose, maltose, trehalose, raffinose, dextrin, glycogen, starch, xylan, mannitol, inositol, inulin, sorbitol, and dulcitol. Glycerol and lactate are attacked very feebly in this medium. However, if 0.8 per cent sodium acetate is also added, both glycerol and lactate are decomposed vigorously. Acid and a moderate amount of gas are formed from the carbohydrates and polyalcohols. The acid is generally a mixture of acetic and butyric acids (see below), and the gas is a mixture of hydrogen and carbon dioxide. Iron milk is slightly acidified without



FIG. 1. CLOSTRIDIUM LACTO-ACETOPHILUM STRAIN 3, FORTY-EIGHT-HOUR CULTURE GROWN IN MEDIUM 1 WITH 0.1 PER CENT AGAR Free spores are visible in the background. × 1,000.

clotting, and a very small amount of gas is formed. Nitrate is not reduced; indole is not formed in a glucose yeast autolysate medium. A little hydrogen sulfide is formed.

Strain 3, which was used for most of the later experimental work, grows at temperatures from 16 to 46 C. The optimum appears to be close to 39 C. The pH range is from 5.6 to 8.4, good growth occurring between pH 6.2 and 7.4. The organisms grow well in a mixed sodium and potassium phosphate buffer in concentrations up to 2 per cent; with 2.4 per cent buffer growth is perceptibly inhibited.

Classification. All the strains studied clearly belong to one species which is evidently closely related to *Clostridium butyricum* as defined by Bergey *et al.*

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However, it is definitely stated that the latter species is unable to fer-(1939).ment lactate. Two lactate-fermenting clostridia have been described in the literature, Clostridium tyrobutyricum of van Beynum and Pette (1935), and Granu*lobacter lactobutyricus* of Beijerinck (1893), but neither of these organisms appears to be able to attack the wide variety of carbohydrates and polyalcohols fermented by our strains. Beijerinck states that G. lactobutyricus is unable to attack carbohydrates at all. C. tyrobutyricum is described as usually fermenting only glucose, fructose, and lactate. In view of the impossibility of identifying our organism with any previously described species, we have decided to call it Clostridium *lacto-acetophilum*, nov. spec., for reasons which will appear below. Further work may prove that this organism is identical with some other species not now recognized to be able to ferment lactate because the fermentation test was carried out in the absence of acetate (see below). However, in the meantime the name C. lacto-acctophilum will serve to identify lactate-fermenting butyric acid bacteria of the type we have described.

Nutritional requirements. Shortly after pure cultures of C. lacto-acetophilum were first isolated, using a solid lactate yeast autolysate medium (medium 1), the organism was inoculated into a liquid medium of the same composition except for the absence of agar. Growth in this liquid medium was extremely sparse. The addition of 0.1 per cent agar resulted in a slight improvement, and the addition of 2 per cent agar allowed moderately good growth. The failure of the organism to grow satisfactorily in liquid medium 1 could not be due to oxygen inhibition since the addition of 0.05 per cent sodium thioglycolate as a reducing agent did not cause any improvement.

An attempt was made to improve the liquid lactate yeast autolysate medium by adding various substances to it, including larger amounts of yeast autolysate. It soon became evident that growth in the liquid medium is almost proportional to the yeast autolysate concentration up to a level of about 30 volumes per cent. This in itself would not be surprising were it not for the fact that 20 volumes per cent yeast autolysate are required to give as good growth in the liquid medium as can be obtained with only 3 volumes per cent in an otherwise identical solid medium.

Previous experiments conducted in this laboratory (Barker, 1947; Bornstein and Barker) with another bacterium, *Clostridium kluyveri*, had shown that an abnormally high requirement for yeast autolysate may be due to a need for acetic acid, which is always present in yeast autolysate in small amounts. We therefore tried adding 0.3 per cent sodium acetate to the liquid lactate medium containing 3 volumes per cent yeast autolysate. The results were very striking; excellent growth occurred in the presence of acetate, whereas in its absence growth was extremely poor. Quantitative experiments on the relation between acetate concentration and growth measured with an Evelyn colorimeter showed that the growth rate and the maximum cell yield increase with sodium acetate concentration up to about 0.8 per cent. Table 1 shows that the total amount of lactate decomposed also increases with the initial acetate concentration.

Several other substances were tested to determine whether they can substitute

for acetate in stimulating growth and lactate decomposition. The substances so tested were formate, propionate, butyrate, fumarate, succinate, malate, tartrate, citrate, pyruvate, ethanol, and glucose. They were used at concentrations of 0.1 and 0.5 per cent. Both lactate and lactate-acetate media were used as controls. It was found that only pyruvate can substitute for acetate in favoring both growth and lactate decomposition. Glucose, either alone or in combination with lactate, supports very good growth, but it does not accelerate the disappearance of lactate.

It should be noted that C. lacto-acetophilum differs markedly from C. kluyveri with respect to the substrates that can be substituted for acetate. The latter organism can use propionate, butyrate, and to a lesser extent valerate, but cannot use pyruvate.

	•		
INITIAL ACETATE	LACTATE DECOMPOSED	RELATIVE GROWTH	
	mu/100 ml		
0.05	0.44	177	
0.79	1.89	446	
1.52	2.89	680	
2.26	3.78	809	
3.00	4.67	888	
3.73	5.22	982	
4.47	5.78	1,107	
5.94	6.22	1,177	
7.40	6.55	1,192	

 TABLE 1

 The dependence of growth and lactate decomposition on the acetate supply

Medium: sodium lactate, 8.8 mm per 100 ml, yeast autolysate, 3 volumes per cent, the salts of medium 1, and the indicated sodium acetate concentrations. All cultures were incubated 3 days at 37 C under anaerobic conditions. Strain 3.

When 0.6 to 0.8 per cent acctate is added to the culture medium, the concentration of yeast autolysate needed to give maximal growth is greatly reduced. About 3 volumes per cent is quite adequate, whereas at lower concentrations the cell yield decreases. However, the yeast autolysate level can be still further reduced to about 0.1 volume per cent without limiting growth if the medium is supplemented with 0.01 μ g biotin and 10 μ g para-aminobenzoic acid per 100 ml. A few attempts to replace yeast autolysate completely by known growth factors and amino acid mixtures were unsuccessful. In a medium containing lactate, acctate, biotin, para-aminobenzoic acid, and 0.01 volume per cent yeast autolysate, the organism failed to respond favorably to any of the following compounds or preparations: thiamine, nicotinic acid, riboflavin, pyridoxine, folic acid, acid-hydrolyzed casein, peptone, and tryptone.

The following medium (medium 2), which supports excellent growth of C. lacto-acetophilum, was developed on the basis of the foregoing experiments: sodium lactate, 1 g; sodium acetate, 0.8 g; yeast autolysate, 0.5 ml (0.05 g dry Fermentation products. The observation that the amount of lactate decomposed is dependent upon the initial acetate concentration suggests that acetate is consumed in the lactate-acetate fermentation. This possibility was verified experimentally, and the products of the lactate-acetate fermentation in a growing culture were determined quantitatively by methods previously described (Barker and Haas, 1944). The main products are butyric acid, carbon dioxide, and hydrogen (table 2, column 3).

The influence of acetate concentration on acetate utilization and butyrate formation is shown in table 3. Only at the lowest initial concentration is there a

TABLE	2
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rermentation products	8	product	on	tati	en	erm.	F
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	ENRICHMENT CULTURE	PURE CULTURE, STRAIN 3			
COMPOUND	Substrates				
	Lactate, 1%	Lactate, 1% + acetate, 0.8%	Pyruvate, 1%	Glucose, 1%	
Acetic acid	50	-32	33	28	
Butyric acid	35	65	33	73	
Carbon dioxide	55.5	100	93	190	
Hydrogen	10	.59	30	182	
Carbon recovery (%)	98	99	97	90	
Redox index	1.39	1.05	0.96	1.12	

The figures represent yields in moles per 100 moles of fermented substrate. Each medium contained the salts of medium 1, 3 volume per cent yeast autolysate, and the indicated substrate concentrations. Cultures were incubated at 37 C until fermentation ceased.

net production of both acetate and butyrate. At higher acetate concentrations there is always a disappearance of acetate, which increases with concentration. It should be noted that added acetate is never entirely used up. The final concentration is always above 1 millimole per 100 ml. In this respect acetate behaves quite differently from most other substrates, like glucose and lactate, which under favorable conditions are completely fermented by this organism. The explanation for this anomalous behavior appears to be that the utilization of acetate depends upon the concentration of butyrate. Column 5, table 3, shows that the butyrate-acetate concentration ratio in the fermented medium never exceeds a value of about 2.6 and is remarkably constant and independent of the initial acetate concentration over a wide range. This indicates the existence of some sort of equilibrium between acetate and butyrate. The ratio of butyrate formed to acetate used (column 6, table 3) is more variable than the final concentration

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ratio. The former ratio is infinite at the lowest acetate level, indicating that all the butyrate is derived from lactate. At intermediate acetate levels, approximately one mole of acetate is used for each mole of butyrate produced. At higher levels, the molar quantity of acetate used considerably exceeds the butyrate formed. Since it is theoretically impossible to use more than one mole of acetate plus one mole of lactate in the formation of one mole of butyrate, this result must mean that some other product, such as acetone, is being formed under these conditions.

The lactate-acetate fermentation is evidently analogous to the ethanol-acetate fermentation of C. *kluyveri*. In the latter process, the ethanol is oxidized to an active form of acetic acid, which condenses with the substrate acetate to give an intermediate compound that is ultimately reduced to butyric acid (Barker,

	тм/1				
	Acetate			FINAL BUTYRATE FINAL ACETATE	BUTYRATE FORMED
Added	Final	Used	- Butyrate formed		
0.05	0.41	-0.36	0.295	0.72	~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
1.27	1.04	0.23	1.24	1.19	5.39
2.49	1.01	1.48	2.23	2.21	1.51
3.76	1.13	2.58	2.95	2.62	1.02
4.93	1.50	3.43	3.90	2.60	1.14
6.15	1.82	4.32	4.74	2.60	1.10
7.37	2.03	5.34	5.27	2.60	0.99
8.59	2.14	6.45	5.55	2.60	0.86
9.81	2.25	7.56	5.85	2.60	0.77
11.03	3.93	7.10	4.72	1.61	0.66
12.25	5.20	7.05	3.47	0.67	0.49

 TABLE 3

 Influence of acetate concentration on the lactate acetate fermentation

Medium no. 1 containing 3 volumes per cent yeast autolysate, 1 per cent sodium lactate, and the specified amount of sodium acetate was used. The cultures were incubated for 5 days at 37 C until fermentation had ceased. Strain 3.

Kamen, and Bornstein, 1945). In the lactate-acetate fermentation of C. lactoacetophilum lactate is evidently oxidized to carbon dioxide and acetic acid or a derivative thereof. It will be noted that one mole of carbon dioxide is formed per mole of lactate fermented as is required of such a mechanism. As in the C. *kluyveri* fermentation, butyric acid is probably formed by a condensation of two moles of acetic acid or related compound, followed by a reduction. The postulated fermentation mechanism may be schematically represented as follows:

$$CH_{3}CHOHCOOH = CH_{3}COOH + CO_{2} + 4H$$
(1)

$$2H = H_2 \tag{2}$$

$$2CH_{3}COOH + 4H = CH_{3}CH_{2}CH_{2}COOH + 2H_{2}O.$$
 (3)

The need for an outside supply of acetate in the decomposition of lactate may be explained by considering the oxidation-reduction relations involved in the fer-

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mentation. These relations are independent of any specific mechanism but for convenience we shall assume the reactions postulated above. The oxidation of 100 moles of lactate to acetate and carbon dioxide (equation 1) releases 400 equivalents of hydrogen, which must be used in reduction processes if the fermentation is to maintain itself. Part of the hydrogen (118 equivalents) appears as hydrogen gas (equation 2). The remainder can only be used in the formation of butyric acid or the reduction of small quantities of hydrogen acceptors present in the yeast autolysate. The latter will be quantitatively important only when the concentration of acetate is low. The reduction of 100 moles of acetate (derived from the oxidation of 100 moles of lactate) to 50 moles of butyrate would consume only 200 equivalents of hydrogen, leaving an excess of 82 equivalents. Extra acetate is required to form a sufficient quantity of a hydrogen acceptor to react with this excess. These relations may be summarized by saying that the fermentation of lactate by this organism cannot proceed without the intervention of an outside oxidizing agent, which in this case is acetic acid. In this connection it should be noted that glycerol also cannot be decomposed by this organism unless acetate is added to act as an oxidizing agent.

Pyruvate and glucose, in contrast to lactate and glycerol, can be fermented in the absence of added acetate (table 2, columns 4, 5). The nonnecessity of extra acetate as an oxidant in the pyruvate fermentation is easily explained. Pyruvate is in a more oxidized state than lactate; when it is converted to acetate and carbon dioxide, two equivalents of hydrogen become available. Since this is just enough hydrogen to make possible the reduction of the acetate simultaneously formed, no outside source of acetate is necessary. In fact, there is a considerable net accumulation of acetate in the fermentation of pyruvate due to the fact that part of the available hydrogen is not used for butyrate formation but is given off as hydrogen gas.

The decomposition of glucose in the absence of added acetate must be explained on a different basis since glucose, unlike pyruvate, is in the same oxidation state as lactate. The fermentation of glucose alone is possible because of the relatively much larger evolution of gaseous hydrogen. In the pyruvate fermentation, involving a two-step oxidation to acetate and carbon dioxide, the yield of hydrogen gas is 30 mm per 100 mm of substrate, and in the lactate fermentation, involving a four-step oxidation, the yield is 59 mm hydrogen, a proportional amount. On the same basis one would expect that the glucose fermentation, which involves eight oxidation steps, would yield about 120 mm hydrogen. However, the observed hydrogen production is 182 mm, about 50 per cent greater than this expectation. From these results one may conclude that some intermediate hydrogen donor in the glucose fermentation, possibly triosephosphate, contributes more heavily to gaseous hydrogen formation than do the compounds involved in the lactate and pyruvate fermentations.

Fermentation of lactate by enrichment cultures. Although added acetate is essential for the fermentation of lactate by pure cultures of C. lacto-acetophilum, this is not true of the first enrichment cultures for this organism. It has been repeatedly observed that a liquid lactate yeast autolysate medium inoculated with

soil supports an excellent growth of anaerobic bacteria, most of which appear to be C. lacto-acetophilum, and the lactate is rather rapidly and completely decomposed. When a transfer is made from the first enrichment culture to the same medium (without acetate), the growth is somewhat poorer and the lactate decomposition is less complete. For example, in one experiment only 82 per cent of the lactate was used up in such a culture, whereas after a second transfer only 26 per cent of the lactate was consumed. These results show that some factor, possibly a second organism, is operating in the first enrichment cultures in such a way as to make the addition of acetate unnecessary. By making successive transfers, this factor is lost and acetate becomes essential just as it is in pure culture experiments.

In order to find what oxidant is substituted for acetate in lactate enrichment cultures, the fermentation products were determined quantitatively (table 2, column 2). The analytical results evidently contain some errors, as evidenced by the high redox index, but they nevertheless are satisfactory to show several striking differences between enrichment and pure culture fermentations. Acetic acid is produced rather than consumed in the enrichment culture, and the yields of butyrate, carbon dioxide, and hydrogen are all greatly reduced. The low yield of carbon dioxide, coupled with the high yield of acetic acid, clearly indicates the utilization of carbon dioxide as an important oxidizing agent in the enrichment culture. The fermentation is very similar to that caused by *Butyribacterium rettgeri* (Barker, Kamen, and Haas, 1945), which has been proved to involve a reduction of carbon dioxide to acetic acid. However, we have not been able to obtain any evidence for the occurrence of species of *Butyribacterium* in the enrichment cultures. The organism or factor responsible for the anomalous behavior of enrichment cultures remains to be discovered.

DISCUSSION

We have shown that a lactate-fermenting butyric acid bacterium, *Clostridium lacto-acetophilum*, can be readily isolated from soil and that the ability of this organism to decompose lactate in pure culture is dependent upon the presence of acetate. The catabolism of this organism may be described as an oxidation of lactate to acetate and carbon dioxide, coupled with a reductive condensation of acetate to butyrate. A little gaseous hydrogen is also formed. Since the amount of acetate produced from lactate is insufficient to react with all the hydrogen derived from lactate, extra acetate must be added to maintain the reaction.

C. lacto-acetophilum is not the only organism requiring acetate as a primary oxidant. A similar type of fermentation is catalyzed by C. kluyveri, which oxidizes ethanol to acetic acid and then converts the acetic acid first to butyric acid and later to caproic acid (Barker, Kamen, and Bornstein, 1945). The catabolic processes of the two organisms differ mainly with respect to the substrates that can be oxidized and the products of acetate condensation and reduction. Experiments with isotope-labeled acetic acid (Wood *et al.*, 1944, 1945; Barker, Kamen, and Haas, 1945) clearly show that other butyric acid bacteria,

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like C. butylicum, C. acetobutylicum, and Butyribacterium rettgeri, also use acetate as a primary oxidant, even though they do not need to have it supplied from an outside source. In such organisms, accessory oxidants, such as carbon dioxide, butyric acid, or acetone, are available to supplement acetic acid. In addition gaseous hydrogen may be formed.

Our results explain why previous investigators have had so little success in growing butyric acid bacteria on lactate media in pure culture. Their bacteria did not lose the ability to ferment lactate, as has commonly been supposed; they simply were unable to oxidize lactate in the absence of acetate. When acetate was unintentionally added, as was done by van Beynum and Pette (1935) through the use of large amounts of yeast extract, the bacteria grew and decomposed lactate. It seems likely that the ability to ferment lactate and glycerol is much more widely distributed among butyric acid bacteria than has been previously reported. At least some species that are now thought to be unable to attack lactate and glycerol will probably be found to do so when acetate is added to the test media. The addition of 0.5 per cent sodium acetate to all media for butyric acid bacteria will probably be found advantageous.

SUMMARY

The isolation and characteristics of a lactate-fermenting butyric acid bacterium, *Clostridium lacto-acetophilum*, nov. spec., are described. It is shown that the decomposition of lactate by this organism is dependent on the presence and simultaneous utilization of acetate. The role of acetate in butyric acid fermentations is discussed.

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