CLOSTRIDIUM RUBRUM SP. N. AND OTHER PECTINOLYTIC CLOSTRIDIA FROM SOIL[1]

HENRY NG AND REESE H. VAUGHN

Department of Food Science and Technology, University of California, Davis, California

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ABSTRACT

NG, HENRY (University of California, Davis) AND REESE H. VAUGHN. *Clostridium rubrum* sp. n. and other pectinolytic clostridia from soil. J. Bacteriol. **85**:1104–1113. 1963.—Reports in the literature and results of experiments described herein suggest that pectinolytic anaerobes constitute a very heterogeneous group. The cultures isolated in this study all belonged to the genus *Clostridium*. The following species were identified: *C. butyricum*, *C. fallax*, *C. multifermentans*, and *C. indolis*. In addition, a species believed to be previously undescribed was named *C. rubrum* sp. n. The ability to ferment galacturonic acid was found to be adaptive. Some cultures fermented pectin and pectic acid to the same degree, whereas others fermented pectin only partially. The partial fermentation was attributed to the lack of a pectinesterase. On the basis of fermentation balances, it was concluded that the four strains of galacturonic acid fermenters selected for study yielded identical end products in approximately the same proportions. Per mole of galacturonic acid fermented, about 2 moles of CO_2, 1.5 moles of H_2, 1.5 moles of acetic acid, and 0.25 mole of butyric acid were produced.

The existence of anaerobic pectinolytic bacteria is well documented, particularly in connection with the retting of fibrous plants used for textiles, where these organisms serve to remove the pectic substances from the middle lamellae of the plants, leaving the cellulose fibers intact. The taxonomic aspects of this group were thoroughly discussed by Raynaud (1949) and more recently by Lanigan (1959). Despite the numerous reports on the occurrence of this group of bacteria, the nature of their metabolism of pectic substances is not well understood. This investigation provides additional information in this area and concerns the isolation, characterization, and classification of some pectinolytic, anaerobic sporeforming bacteria with special reference to their activity on a variety of pectic substances.

[1] This investigation constitutes part of a Master of Science thesis submitted by the senior author to the Graduate Division of the University of California, Davis.

MATERIALS AND METHODS

Organisms. For the purpose of comparing the cultures isolated in the present study, several strains of *Clostridium* were obtained from the following sources: *C. felsineum* strains 546 and 638, *C. roseum* 653, and two other unnamed pigmented clostridia, cultures 596 and 599, all through the courtesy of L. S. McClung of Indiana University, Bloomington; *C. corallinum* strains BBII and TIR and *C. saturnirubrum* A6D were obtained through the courtesy of A.-R. Prévot, Institut Pasteur, Paris, France.

Chemicals. α-D-Galacturonic acid, pectic acid, pectin, and sodium polypectate were obtained from Sunkist Growers, Ontario, Calif. The galacturonic acid was recrystallized from acetone in a refrigerator and then dried as the monohydrate under vacuum. The rest were used as received. Other chemicals were of reagent grade obtained from the usual sources.

Bacteriological media. All media were adjusted to pH 7.0 with 1 N NaOH and sterilized by autoclaving at 120 C for 15 min. Solid media were prepared by addition of 2% agar to liquid media. These general procedures were adhered to, except where noted.

Sodium polypectate medium was prepared by blending to a creamy mixture in a Waring Blendor in 500 ml of cold distilled water, followed by the addition of 500 ml of boiling water: Tryptone, 5 g; proteose peptone, 5 g; soluble starch, 1 g; sodium thioglycolate, 0.5 g; Calgon (food grade), 2.5 g; sorbic acid, 1 g; and sodium polypectate, 60 g. The medium, which had a pH of about 6.5, was then sterilized in flasks filled to about one-fourth of capacity.

Pea infusion broth was made by dissolving 5 g of yeast extract, 10 g of Tryptone, 1 g of soluble starch, 1 g of K_2HPO_4, and 0.5 g of sodium thioglycolate in 500 ml of distilled water and 500 ml of pea infusion (double strength) which was prepared according to the procedure given by the National Canners Association (1956). Liver infusion broth had the following composition: Tryptone, 5 g; K_2HPO_4, 1 g; distilled water, 500 ml; and liver infusion (double strength), 500 ml. The liver infusion was prepared by simmering ground, fat-free beef liver in an equal weight of distilled water for 1 hr. The infusion was first filtered through cheese cloth to remove the large particles of liver. Then it was passed through filter paper using diatomaceous earth as a filter aid. The filtrate was then made up to its original volume. The liver particles were dried, and a small amount was added to tubes of liver infusion broth along with a pinch of $CaCO_3$.

Corn infusion broth was prepared by boiling 50 g of dried ground yellow corn in 1 liter of distilled water which was then filtered through cheese cloth to remove the larger particles. The filtrate was made up to its original volume to correct for evaporation. This medium had a pH of about 6.5 after autoclaving. The criterion for fermentation of a carbohydrate was the ability to produce acid and gas in a basal medium of the following composition (per liter): test substrate, 10 g; proteose peptone, 10 g; Tryptone, 10 g; yeast extract, 5 g; sodium thioglycolate, 2 g; K_2HPO_4, 1 g; and agar, 2.5 g. The same medium minus the test substrate served as the control. Liver-gelatin consisted of double-strength liver infusion diluted with an equal volume of distilled water. Then 12% (w/v) gelatin was added to the mixture. The medium for sporulation was the same as that used for fermentation tests, except that the test substrate was replaced by 1% (w/v) soluble starch. The test for ability to digest coagulated albumen was carried out in a liver infusion broth to which was added approximately 1 cm^3 of heat-coagulated egg white. The media and methods used for determination of indole production (Kovac's test), nitrate reduction, H_2S production (0.02% lead acetate in the basal medium), and reaction to litmus milk were those described in the *Manual of Microbiological Methods* (Society of American Bacteriologists, 1957). The anaerobic buffer had the following composition: Na_2S (added just prior to use), 0.5 g; K_2HPO_4, 0.1 g; KH_2PO_4, 0.1 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $MnSO_4 \cdot 3H_2O$, 0.01 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; NaCl, 0.01 g; and distilled water, 1,000 ml.

Experimental procedures. The sodium polypectate gel described above was used as the enrichment medium for the isolation of pectinolytic species of *Clostridium*. This medium contained soluble starch which served to overcome dormancy of spores (Wynne and Foster, 1948) and sorbic acid to inhibit catalase-positive organisms (York and Vaughn, 1954). To eliminate the nonsporeformers, the soil samples (1 g), in duplicate, were suspended in 1 ml of water in test tubes (25 by 200 mm), pasteurized in a water bath at 85 C for 5 min, and cooled. After addition of 0.6 ml of filter-sterilized, 10% $NaHCO_3$, about 30 ml of the polypectate medium, cooled to 60 to 70 C, were poured into the tubes containing the pasteurized soil suspensions. The tubes were then sealed with Vaspar to inhibit the strict aerobes. When the tubes had cooled to room temperature and the gel had solidified, the cultures were incubated at 35 C. Liquefaction of the polypectate gel constituted a positive enrichment. All positive enrichments were carried through the enrichment procedure three times to assure the predominance of the pectinolytic flora.

Isolation of the pectinolytic clostridia was accomplished by transferring a sample of the enrichment culture into pea infusion broth. When spores were observed in this medium (usually after 3 to 5 days), one drop of each culture was streaked out on pea infusion agar in plates having porous ceramic tops. The plates were incubated at 35 C, under a nitrogen atmosphere, in anaerobic jars. Just before sealing, an open petri dish containing 6 g of pyrogallic acid and 24 ml of 20% Na_2CO_3 was placed at the bottom of the jar. In addition, a tube of methylene blue indicator, prepared according to the directions given in the *Manual of Microbiological Methods* (Society of American Bacteriologists, 1957) was included to serve as an indicator of anaerobiosis.

Generally, the media for liquid cultures were cooled and inoculated immediately after autoclaving. Tubes of media not used soon after sterilization were immersed for 20 min in a boiling-water bath to drive off dissolved oxygen. Re-entrance of air into inoculated tubes was prevented by a Vaspar seal.

Facultative anaerobes were culled from the cultures of strict anaerobes by streaking pure, sporulated pea infusion cultures onto pea infusion agar plates in duplicate. One set was incubated

anaerobically and a duplicate set aerobically at 35 C.

Cellular morphology, including motility, spore form, and position was determined by phase microscopy on wet mounts sealed under a cover slip with Vaspar. Cell size was determined by use of a calibrated eyepiece. Cultures for spore form and position were grown in pea infusion or corn infusion broth, depending on which medium yielded a higher percentage of discrete sporangia. Cultures for cell shape, size, motility, and Gram reaction were grown in liver infusion broth for 12 hr. Gram stains were performed by use of the Hucker modification as described by the Society of American Bacteriologists (1957). As controls, smears of *Lactobacillus plantarum* and *Escherichia coli* were placed on the same slide with the smears of the test organisms.

The production of pigment was tested on potato, corn, and pea infusion broths and agars.

The rates of pectin and pectic acid fermentation by *C. felsineum* 638 and 546 were determined in a basal medium containing 1% (w/v) pectin or pectic acid. Tubes containing 20 ml of medium were inoculated with 2 ml of an 18 hr liver infusion-1% galacturonic acid culture and incubated at 37 C. At 0, 4, 8, 12, 24, and 48 hr, tubes were removed from the incubator and the following determined: pH, titratable acidity, total anhydrogalacturonic acid, and free monogalacturonic acid.

The adaptive utilization of galacturonic acid by *C. felsineum* 546 was demonstrated by the following two methods: evolution of gas was measured manometrically by conventional Warburg techniques (Umbreit, Burris, and Stauffer, 1957) under the conditions stated in the legend of Fig. 2; and disappearance of galacturonic acid in a basal medium incubated at 37 C for 150 min as described in Table 6. Cells used in both experiments were grown in 225 ml of liver infusion broth with and without 0.1% galacturonic acid in 250-ml Florence flasks. When gas evolution began to subside as measured by the displacement of water from a flask into a graduated cylinder, the culture was harvested by centrifugation, and the cells were resuspended in anaerobic buffer.

Carbon balances of galacturonic acid fermentation by four of the cultures studied (*C. felsineum*, strains 546 and 638, culture numbers 34 and 22) were done in 30-ml volumes in large (approximately 130 ml) Warburg vessels with mercury manometers. Since these vessels lacked center wells, parallel fermentations of 1:10 in scale were run in conventional Warburg vessels to determine the ratio of CO_2 to H_2. This ratio was applied to the total gas produced in the large vessels in calculating the amount of CO_2 and H_2 produced. The adapted cells used for these fermentations were grown and harvested as described above. Although the cells were not washed, little growth could have occurred, as indicated by the almost constant rate of gas evolution. Other conditions of the experiment are stated in Table 7. These fermentations were usually completed in about 12 hr or less, as indicated by cessation of gas evolution. After the cells were removed by centrifugation, the supernatant fluid and washings were pooled, made up to a known volume, and analyzed.

Chemical methods. By the carbazole method of McComb and McCready (1952), pectin and pectic acid were analyzed and expressed as anhydrogalacturonic acid. Galacturonic acid, in the absence of pectin or pectic acid, can also be determined by this method. However, when pectin or pectic acid was present, galacturonic acid had to be determined selectively by the naphthoresorcinol method of Mills (1951). Glucose was estimated by the reducing group reaction with potassium ferricyanide, as described by Schales and Schales (1945). Total acids were qualitatively and quantitatively determined on 2 ml of appropriately concentrated culture supernatant fluid by partition chromatography (Wiseman and Irvin, 1957). The identity of the acids was confirmed by paper chromatography (Brown and Hall, 1950). Volatile acids were determined by steam distillation followed by partition chromatography as described for total acids. Acetylmethylcarbinol, glycerol, and 2,3-butylene glycol were determined by the methods of Neish (1952).

RESULTS

Characteristics of the isolates. Of 31 obligate anaerobes isolated, 17 were able to liquefy the polypectate gel to varying degrees within 96 hr (Table 1). Unfortunately, three of these were lost during the course of the study. The remaining 14 cultures were found to have retained their liquefying properties, even 2 years after isolation. All the cultures grew well at 35 C but not at 55 C, despite incubation at the higher temperature for 1 month. All the cultures, as expected, were sporeformers, since the soil from which they

were isolated had been pasteurized. The isolates were allocated into three groups (Table 2). Group 1 consisted of large (0.9 by 6.3 to 23 μ), gram-positive, motile rods with subterminal oval spores. Group 2 had smaller (0.6 by 4.4 to 4.8 μ), actively motile, gram-negative rods with terminal spherical spores. The group 3 isolates, represented by two cultures, were similar to group 1 except for eccentrically located spores and production of a pigment which varied from a pale pink to light red, depending on nutritional conditions (Table 3). All three groups had swollen sporangia. Their fermentation and other biochemical characteristics are summarized in Tables 4 and 5.

TABLE 1. *Pectinolytic activities of isolates**

Culture no.	Polypectate liquefaction†	Percentage of substrate fermented		
		Pectin	Pectic acid	Galacturonic acid
1	+	70	82	12
5	++	30	79	53
6	++	24	77	10
30	++	26	82	70
33	++	78	79	67
22	++	3	3	86
7	++	21	69	80
9	+	24	73	80
12	++	21	72	68
23a	+	22	69	86
24a	++	21	70	81
28b	++	21	71	84
34	+	0	6	86
35	++	3	3	87
638	+++	82	93	86
546	+++	78	92	86

* Cultures were incubated at 37 C for 4 days.
† Symbols: + = slight, ++ = fair, and +++ = extensive.

Rate of pectin and pectic acid fermentation. C. felsineum strains 546 and 638 fermented 1% pectin and pectic acid almost completely within 24 hr. At the same time, it was found that there was a corresponding increase in titratable acidity and a drop in pH to between 5.2 and 4.8. However, there was no appreciable accumulation of free monogalacturonic acid in the medium. Since both strains gave almost identical results, only those obtained with strain 546 are shown in Fig. 1.

Adaptive utilization of galacturonic acid. That the ability to use galacturonic acid is an adaptive process can be seen in Fig. 2. Cells grown on liver infusion broth without galacturonic acid could not use galacturonic acid but could ferment glucose, whereas cells grown in the presence of galac-

TABLE 2. *Morphological characteristics of isolates*

Culture no.	Spore position*	Spore shape*	Sporangium swollen	Motility	Gram reaction	Species allocation
Group 1						
1	st	ov	+	+	+	*C. multifermentans*
5	st	ov	+	+	+	*C. butyricum*
6	st	ov	+	+	+	*C. butyricum*
30	st	ov	+	+	+	*C. butyricum*
33	st	ov	+	+	+	*C. butyricum*
22	st	ov	+	+	+	*C. fallax*
Group 2						
7	t	sph	+	+	−	*C. indolis*
9	t	sph	+	+	−	*C. indolis*
12	t	sph	+	+	−	*C. indolis*
23a	t	sph	+	+	−	*C. indolis*
24a	t	sph	+	+	−	*C. indolis*
28b	t	sph	+	+	−	*C. indolis*
Group 3						
34	ec	ov	+	+	+	*C. rubrum* sp. n.
35	ec	ov	+	+	+	*C. rubrum* sp. n.
Clostridium felsineum						
638	st	ov	+	+	+	
546	st	ov	+	+	+	

* Abbreviations: st = subterminal, t = terminal, ec = eccentric, ov = oval; sph = spherical.

TABLE 3. *Comparison of color of pigment of cultures 34 and 35 with known cultures on various media*

Culture no.	Potato infusion		Pea infusion		Corn infusion	
	Broth	Agar	Broth*	Agar	Broth	Agar
596	Red-orange	X†	Orange	X	Orange	Orange
599	Red-orange	Orange	Orange	Orange	Orange	Orange
653	Orange	X	Orange	Colorless	Colorless	Orange
546	Red-orange	Orange	Colorless	Orange	Yellow	Orange-yellow
638	Red-orange	X	Colorless	Orange	Yellow	Orange
34	Light red	Light red	Pink	Light red	Colorless	Pale pink
35	Light red	Light red	Pink	Light red	Colorless	Pale pink
BBII	X	X	Colorless	Colorless	X	X
TIR	X	X	Colorless	Colorless	X	X
A6D	X	X	X	X	X	X

* Color shown is for color of sediment in pea infusion broth.
† Spores failed to germinate.

TABLE 4. *Saccharolytic characteristics of isolates**

Culture no.	Substrate						
	Glucose	Lactose	Sucrose	Starch	Mannitol	Glycerol	Cellulose
Group 1							
1	+	+	+	+	−	+	−
5	+	+	+	+	−	−	−
6	+	+	+	+	−	−	−
30	+	+	+	+	−	−	−
33	+	+	+	+	−	−	−
22	+	+	+	+	−	−	−
Group 2							
7	+	+	+	−	+	−	−
9	+	+	+	−	+	−	−
12	+	+	+	−	+	−	−
23a	+	+	+	−	+	−	−
24a	+	+	+	−	+	−	−
28b	+	+	+	−	+	−	−
Group 3							
34	+	+	+	+	+	−	−
35	+	+	+	+	+	−	−
Clostridium felsineum							
638	+	+	+	+	−	−	−
546	+	+	+	+	−	−	−

* Cultures were incubated at 37 C for 4 days; + = acid and gas, − = not fermented.

turonic acid used either substrate immediately. In another experiment, in which adapted and nonadapted cells were incubated for 150 min at 37 C in the presence of either glucose or galacturonic acid, it was found that galacturonic acid-adapted cells used about 70% of the galacturonic acid, whereas the nonadapted cells used none. Both adapted and nonadapted cells were able to use glucose, although the adapted used considerably less within the time of incubation than the nonadapted cells (Table 6). This apparent discrepancy can be explained by the slower rate of glucose utilization by adapted cells as indicated in Fig. 2.

End products of galacturonic acid fermentation. The end products recovered in the fermentation of galacturonic acid by four strains of pectinolytic clostridia were both qualitatively and quantitatively similar. It can be seen in Table 7 that only CO_2, H_2, and acetic and butyric acids were produced in appreciable quantities. Although the carbon recovery was very good, the oxidation-reduction (O/R) index left much to be desired. Since the method of estimating the gases was not highly refined, it is conceivable that the H_2 values might be a little high, thereby accounting for the low O/R index. However, the results clearly indicated that no major end products had been omitted.

DISCUSSION

As mentioned previously, all the cultures isolated belonged to the genus *Clostridium*, when classified and identified according to *Bergey's Manual* (Breed, Murray, and Smith, 1957). Furthermore, they could be divided into three distinct groups.

Group 1, represented by six isolates, consisted of nonpigmented organisms that possessed oval

TABLE 5. *Other biochemical characteristic of isolates*

Culture no.	Liver-gelatin	Litmus milk*	Coaggulated egg albumen†	Indole production	Nitrite from nitrate	H_2S production
Group 1						
1	−	+	−	−	−	−
5	−	+	−	−	−	−
6	−	+	−	−	−	−
30	−	+	−	−	−	−
33	−	+	−	−	−	−
22	−	±	−	−	−	−
Group 2						
7	−	−	−	+	+	−
9	−	−	−	+	+	−
12	−	−	−	+	+	−
23a	−	−	−	+	+	−
24a	−	−	−	+	+	−
28b	−	−	−	+	+	−
Group 3						
34	−	−	−	−	−	−
35	−	−	−	−	−	−
Clostridium felsineum						
638	+	+	±	−	−	−
546	+	+	±	−	−	−

* Active coagulation, +; slow coagulation, ±; no coagulation, −.
† The ± indicates that the material was softened but not digested.

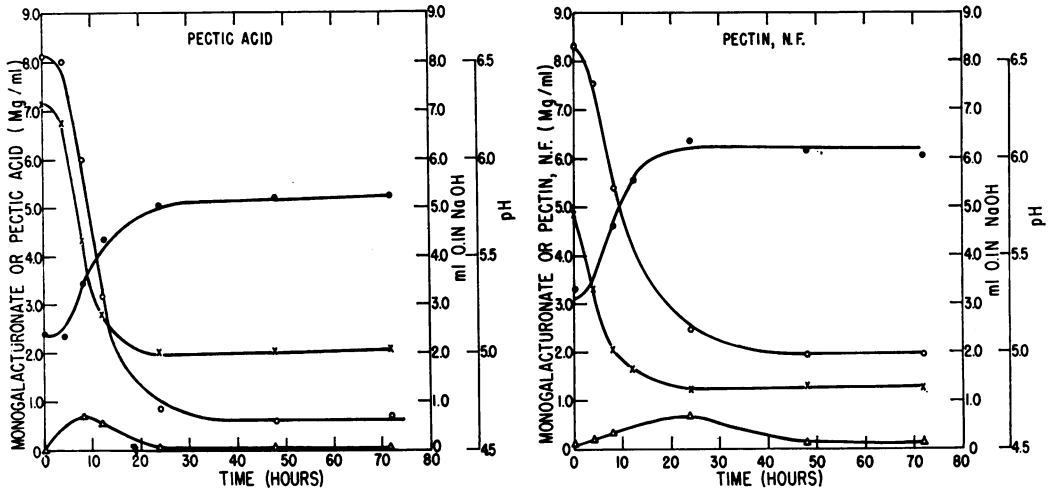

FIG. 1. *Rate of pectic acid and pectin fermentation by Clostridium felsineum 546. Each point represents a determination on replicate cultures sampled at the time indicated. Growth medium was a basal medium containing 1% pectic acid or pectin.* ○ = *Substrate (pectin or pectic acid) remaining;* ● = *titratable acidity;* × = *pH; and* △ = *monogalacturonates accumulating.*

spores. Four of these cultures (5, 6, 30, and 33) were identified as *C. butyricum* Prazmowski. The only deviation from the description given in *Bergey's Manual* for this species was their ability to ferment pectin. This fact is not at all disturbing since a special effort had been made to select for pectinolytic strains. Previously, McCoy and Peterson (1928) screened a total of 45 butyric and butyl anaerobes and were unable to demonstrate this property in any of them. Culture 1 was

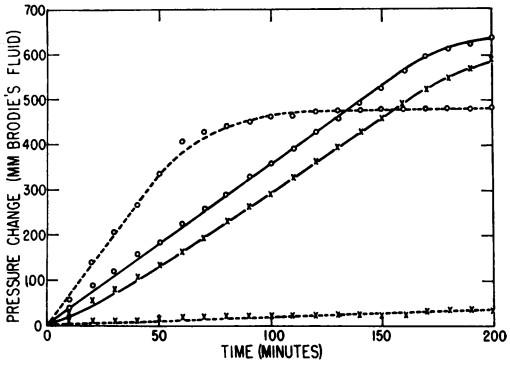

FIG 2. *Manometric experiment showing the adaptive utilization of galacturonic acid by Clostridium felsineum 546. The main compartment of the vessels contained 1.5 ml of anaerobic buffer and 1.0 ml of cells. The center wells contained 0.2 ml of distilled water and the side arms had 10 μmoles of glucose (○) or 10 μmoles of galacturonic acid (×) per 0.5 ml. The solid lines indicate adapted cells and the dotted lines unadapted cells. The experiment was conducted at 30 C under an N_2 atmosphere. The results are corrected for endogenous activity.*

similar to *C. butyricum* in many respects, except that it had the additional ability to ferment glycerol. On this basis, the culture was identified as *C. multifermentans*. The slow coagulation of milk by culture 22 allowed it to be allocated to the species *C. fallax*.

Group 2, containing the cultures with terminal spherical spores, were also unable to produce pigments. The most distinguishing feature of this group was its ability to produce indole from tryptophan, and nitrite from nitrate. Placing the main emphasis on indole formation, these were all identified as *C. indolis*. They differed from the description given for this species in *Bergey's Manual* (Breed et al., 1957) in that they did not coagulate milk and did not produce H_2S. Since the latter character can vary depending on the conditions used, it does not seem justified to create a new species just on the basis of the lack of ability to coagulate milk.

Group 3 cultures 34 and 35 were characterized by their ability to produce a nondiffusible red-

TABLE 6. *Adaptive nature of galacturonic acid utilization by Clostridium felsineum 546, as shown by disappearance of substrate**

Growth medium	Test substrate	Test substrate concn (mg/ml)		
		Initial	Remaining	Utilized
Liver infusion broth	Glucose	7.8	1.9	5.9
	Galacturonic acid	7.6	8.0	−0.4
Liver-galacturonic acid broth	Glucose	8.0	6.7	1.3
	Galacturonic acid	8.4	2.5	5.9

* The incubation mixture consisted of 5 ml of cells in anaerobic buffer and 1 ml of 5% (w/v) glucose or galacturonic acid. The test tubes were sealed with Vaspar and incubated at 37 C for 150 min. The reaction was stopped by heating in boiling water for 5 min.

TABLE 7. *End products of galacturonic acid fermentation**

End products	*Clostridium felsineum* 546	*C. felsineum* 638	*C. rubrum* 34	*C. fallax* 22
Galacturonic acid fermented	235.3	239.6	218.8	230.7
Products				
CO_2	429.9 (182)†	454.2 (190)	422.3 (193)	459.6 (199)
H_2	398.6 (169)	412.6 (172)	396.7 (181)	304.7 (132)
Acetic acid	351.1 (149)	370.6 (155)	331.5 (132)	371.3 (161)
Butyric acid	59.7 (25)	58.7 (25)	48.5 (22)	42.8 (19)
Per cent carbon recovered	97	99	97	99
Oxidation/reduction index	0.87	0.90	0.92	1.08

* Each vessel contained 25 ml of cells suspended in anaerobic buffer to a density of about 5 mg of cells/ml (dry weight). The side arms had 250 μmoles of galacturonic acid in 5 ml of distilled water. The fermentations were conducted under N_2 at 30 C.

† First figure denotes μmoles involved; parenthetical figure indicates moles/100 moles of galacturonic acid fermented.

dish pigment. This pigment was formed only by the cells located at the center of colonies growing on the surface of pea, potato, or corn infusion agar plates. The peripheries of the colonies were a dull gray. The color of the pigment produced on pea and potato infusion agar was a light red, whereas on corn infusion agar the color was a pale pink. In broth cultures, the color could only be seen in potato infusion. In pea infusion, a pink sediment could be observed but in corn infusion the color was so pale that it appeared almost colorless. These organisms produced eccentrically located oval spores. Of the three pigmented nongelatinolytic species described in *Bergey's Manual*, *C. saturnirubrum* Prévot appears to be the most closely related to cultures 34 and 35. Since the culture of *C. saturnirubrum* so kindly furnished by A.-R. Prévot was not viable in the hands of the present authors, a comparative study could not be made. However, the pigment produced by cultures 34 and 35 on three different media, whether in broth or on agar plates, was so different from the saturn red color in the *Dictionary of Color* (Maerz and Paul, 1950) that it is doubtful that the cultures are the same. Prévot (1946) stated that *C. saturnirubrum* does not produce the pigment at 37 C, but at 26 C the colonies are first yellow and then turn saturn red. The two cultures of *C. corallinum*, obtained from A.-R. Prévot, were viable but did not appear to be the same as the cultures which had been described (Prévot and Raynaud, 1944). They were highly proteolytic, and pigment was not formed under the conditions used; therefore, no comparison could be made. It is highly unlikely that the unknown cultures could be *C. corallinum* because the color produced was clearly not coral. *C. belfantii*, also nongelatinolytic, differed from cultures 34 and 35 not only in the color of the pigment formed but in three other characteristics: namely, milk coagulation, indole production, and a weak starch fermentation. The remaining nongelatin liquefier, *C. venturellii*, differed from cultures 34 and 35 in that a rose-colored pigment was produced (only below 25 C), mannitol was not fermented, and milk was coagulated. If the ability to liquefy gelatin could be ignored, cultures 34 and 35 might possibly be identified as *C. roseum*. However, after comparing the culture of *C. roseum* obtained from L. S. McClung, it was found that the color of the pigment produced by this culture was at best an orange-red. Under all the conditions used, the pigment produced by the culture of *C. roseum* was not at all similar to the color of that produced by these two cultures. Furthermore, *C. roseum* seemed to be fairly proteolytic because coagulated egg albumen was softened. The colors of the two unnamed pigmented anaerobes obtained from L. S. McClung also failed to match the color of the two unknown isolates. Having failed to find in the literature a description of a species of *Clostridium* that corresponded to the characteristics of these isolates, the name *C. rubrum* sp. n. is proposed for cultures 34 and 35. However, further study of these isolates may be necessary to determine their exact taxonomic position. Spore suspensions of these two cultures dried on sterile soil have been deposited with the American Type Culture Collection and added to the collection of L. S. McClung of Indiana University.

It is interesting to note that cultures 1 and 6 were able to ferment pectin and pectic acid but could not use galacturonic acid. Since any explanation offered to clarify this anomaly would be pure speculation, it is not dealt with here. The fact that some cultures fermented only about 20% of the pectin but were able to ferment up to 80% of the pectic acid suggests that these organisms possessed a polygalacturonase but not a pectin esterase or a polymethylgalacturonase.

A 1% pectin or pectic acid medium was shown to be fermented to a constant level within 24 hr by *C. felsineum* strains 546 and 638. Potter and McCoy (1952) showed that during the fermentation of pectic acid, but not pectin, by another strain of the same species, an appreciable quantity of galacturonate accumulated. This observation was not confirmed in experiments reported here. This discrepancy might be explained by the fact that the concentration of substrates used in this study was 1% as compared with 2% used by Potter and McCoy (1952) or, more likely, that there are strain differences with respect to this property. Kraght and Starr (1953), using a 1% pectin medium, demonstrated no accumulation of monogalacturonates when fermented by *Erwinia carotovora*.

Although the breakdown of pectin and pectic acid has been shown by many workers to be brought about by an inducible enzyme system, the adaptive nature of galacturonate utilization has not been as clearly demonstrated. Kraght and Starr (1952) showed that the metabolism of galacturonate by *E. carotovora* was adaptive.

Both the manometric data shown in Fig. 1 and the substrate disappearance experiment (Table 6) establish that *C. felsineum* also metabolizes galacturonic acid adaptively.

To the authors' knowledge, no quantitative study has ever been undertaken to determine the end products of fermentation of galacturonic acid by obligate anaerobes. Kraght and Starr (1952) did make a comparative study of the end products of fermentation of glucose and galacturonic acid by a facultative anaerobe, *E. carotovora*. However, they used a nonhydrogen-producing strain which they admitted might be atypical for this species. At any rate, they reported that the end products of galacturonate fermentation were more highly oxidized than those of glucose fermentation (e.g., ethanol production was completely suppressed on the former substrate). Kay (1926) also found that, when a highly oxidized substrate was used by coliform organisms, there was a diminished production of alcohols and an increase in acetic and succinic acids. Johnson, Peterson, and Fred (1931), in a study of the acetone butanol fermentation, compared the fermentation of mannitol, glucose, and calcium gluconate by *C. acetobutylicum* and found that the ratio of acetic acid (redox index = 0) to butyric acid (redox index = −4) increased with increasing oxidation state of the substrate. Enebo, Carlsson, and Lundin (1947) studied the fermentation of purified flax pectin by *C. felsineum* and *Granulobacter pectinovorum* and recovered only acetic and butyric acids. Unfortunately the gases were not analyzed. In the present study, it was found that the end products of galacturonic acid fermentation by four strains of pectinolytic anaerobes were quantitatively and qualitatively similar. In addition to acetic and butyric acids, CO_2 and H_2 were produced. The molar relationships of the dissimilation may be represented by the following balanced empirical equation:

$$\underset{\text{galacturonic acid}}{C_6H_{10}O_7} + 0.5H_2O \rightarrow 2CO_2 + 1.5H_2 + 1.5\underset{\text{acetic acid}}{C_2H_4O_2} + 0.25\underset{\text{butyric acid}}{C_4H_8O_2}$$

As expected, the neutral volatile products, such as acetone, butanol, and other alcohols, which are normally found in glucose fermentations (*see* Lanigan, 1959) are notably absent in galacturonate fermentations. The data do not warrant the speculation as to the pathway of formation of these end products; however, it may be that the early steps are similar to those shown by Kilgore and Starr (1959) for *E. carotovora*. At least the data are consistent with the breakdown via this pathway. Only the isolation of the enzyme systems involved and labeling experiments can clarify this area.

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