Clostridium aurantibutyricum (n.sp.): A Pink Butyric Acid *Clostridium*

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SUMMARY: A pink, butyric acid-producing *Clostridium* isolated by Ch. Weizmann from South African hibiscus stems is described. It ferments sucrose, glucose, lactose, maltose, galactose, xylose, starch, but not cellulose, inulin, mannitol, glycerol and sorbitol.

The main products of fermentation of maize and glucose are butyric and acetic acids; acetone, butanol, ethanol and isopropanol are produced in slight or moderate amounts, lactic acid, formic acid and acetylmethyl-carbinol only in slight or negligible amounts. In its partial diastatic action on maize mashes and in the composition of its resultant metabolic products the new organism resembles members of the so-called butyric group of clostridia; but it differs markedly in liquefying gelatin.

In certain morphological and physiological features the organism resembles two orange-coloured clostridia, *Cl. felsineum* and *Cl. roseum* (both belonging to the so-called butylic group), but is readily distinguishable from them. The two butylic organisms have a complete diastatic action on maize and butanol is the chief product of fermentation. The new organism is also distinguishable by its inability to ferment inulin and to disintegrate potato slopes.

On the basis of this evidence the organism is considered to be a new species, for which the name *Cl. aurantibutyricum* is suggested.

A pink organism, isolated by Ch. Weizmann in May 1939 from a sample of South African hibiscus stems, produced a distinct red coloration on maize mash, gave red colonies on nutrient glucose agar and produced butyric and acetic acids, and small quantities of neutral solvents from sugar, for the complete fermentation of which chalk was required. It produced spores which withstood 2 min. heating at 100°.

A number of these cultures, designated W 45, was investigated after they had been sealed for six to seven months. Some were still viable when subcultured in yeast glucose medium at 37°, but none survived heating for 1–2 min. at 100°, nor for several minutes at 80°. Cultures in maize mash showed active gassing, a definite pink coloration and a good although incomplete diastatic action. The so-called head of solid particles of mash, brought to the surface by the active evolution of gas, sank when the gassing had subsided. The cultures consisted of actively motile rods and clostridia bearing subterminal spores. On yeast glucose agar slopes under anaerobic conditions more or less regular, orange-pink granular colonies were produced (Pl. 1, A). Preliminary experiments showed that the organism dissimilated carbohydrates, producing good yields of butyric and acetic acids and slight amounts of neutral volatile products, such as acetone, butanol, ethanol and isopropanol. These data, and especially the prevailing acidic nature of the fermentation products, tend to

align the pink organism with the so-called butyric clostridia rather than with the butylic bacteria.

Chromogenic clostridia are not unknown. Two red chromogens have been described, viz. *Clostridium felsineum* Carbone & Tomolato (Carbone & Tomolato, 1917) a true retting organism from retting flax, and *Cl. roseum* McCoy & McClung (McCoy & McClung, 1935), found occasionally with *Cl. acetobutylicum*. Because of their special ability to produce high yields of acetone and butanol from starch mashes these two chromogens are grouped with the butylic organisms. No record of a pink butyric organism could be found in the literature except one by McClung (1942) who reported the isolation of some pigmented butyric types from soil and mud. This, naturally, stimulated a close study of the morphology, cultural aspects and physiological properties of the new pink butyric *Clostridium*, with special reference to its taxonomy and nomenclature.

DESCRIPTION OF THE ORGANISM

Morphology

Vegetative cells. Motile, medium and long rods; rounded ends; singly and in short or fairly long chains. Spore-bearing cells motile, mostly spindle-shaped (Pl. 1, B). Good sporulation at 30° on 5% maize, but scanty at 37°, becoming negligible after several subculturings. Tendency for better sporulation on 1% than 5% maize at 37°.

Endospores. Subterminal and oval.

Table 1. *Measurements of vegetative cells, etc., on yeast glucose agar*

	Limits of size		Average size	
	Length (μ .)	Width (μ .)	Length (μ .)	Length (μ .)
Rods:				
(1) 30°	2.8- 8.0	0.55-0.6	4.7	0.58
(2) 37°	4.0-17.0	0.5-0.55	9.4	0.51
Clostridia	5.5-10.5	0.9-1.1	6.9	1.06
Spores	1.9- 2.4	0.85-1.1	2.18	0.97

Flagella. Peritrichous.

Staining reactions. Young cultures Gram-positive. Lugol's iodine: young vegetative cells stain yellow, granules in clostridia stains blue-violet.

Fermentation of carbohydrates and related substances

The ability of the organism to utilize various substances as a source of carbon was determined by measuring the amount of gas and volatile acid produced from 25 ml. of medium containing 20% yeast water, 0.5 g. of the substance under test, and 0.5 g. calcium carbonate in long narrow tubes (23 × 1.7 cm.) connected with water-filled gas burettes. The results are shown in Table 2.

Table 2. *Fermentation of various carbohydrates, alcohols and glucosides (7 days at 37° and 30°)*

	Gas production (ml.)		Volatile acid (ml. 0.1 N)	
	37°	30°	37°	30°
Glucose, lactose, sucrose, maltose, galactose, levulose, arabinose, xylose, mannose, starch	195-270	185-300	27.5-42.5	29-41.5
Raffinose, rhamnose, dextrin	105-120	95-160	28-35.5	22-27.5
Salicin, pectin (0.3 g.)	47-73	42-97	18-18.5	14-17
Glycerol, inulin, glycogen, mannitol, sorbitol, erythritol, cellulose	0-5	0	3-6	1-6.5
Basal medium + inoculum	—	—	3.5	6.0

Fermentation of glucose. Analyses of the fermentation products of glucose in the presence and absence of calcium carbonate are given in Table 3. The inoculant was an active 24 hr. maize-tube culture. In the first series, 2% glucose was added to 600 ml. of 0.5% Difco yeast extract; in the second series 800 ml. of the same medium were taken, whereas in the third and fourth series 3% glucose was added to 600 ml. of yeast medium with the addition of 20 g. of separately sterilized calcium carbonate.

Table 3. *Products of fermentation of yeast glucose medium after 6-7 days' incubation at 37°*

	Without CaCO ₃		With CaCO ₃	
	I	II	III	IV
Glucose fermented (%)	60.38	45.86	100	100
Titrateable acidity (ml. 0.1 N acid/10 ml.)	6.4	6.89	—	—
Products calculated in mmol./100 mmol. of glucose fermented				
Butyric acid	49.4	60.6	43.5	42.1
Acetic acid	51.4	59.4	47.8	63.2
Lactic acid	0.0	9.4	23.9	7.1
Formic acid	0.3	2.7	0.6	1.4
Acetone	3.2	1.8	1.0	1.5
isoPropanol	5.4	6.2	3.0	1.6
Butanol	11.9	8.5	7.1	7.4
Ethanol	34.2	45.9	15.8	14.6
Acetylmethylcarbinol	5.8	Traces	0.3	0.6

Residual sugar was determined according to Lehmann-Maquetenne-Schoorl (van der Haar, 1920).

Neutral solvents were distilled off from 200 ml. of neutralized fermented medium. 100 ml. of distillate were collected. The acetone content was estimated according to Goodwin's modification (Goodwin, 1920) of Messinger's method; *isopropanol*, by the method of Langlykke, Peterson & McCoy (1935) and ethanol and butanol by Johnson's (1932) method.

Volatile acids were determined from 50 ml. of the fermented medium acidified with phosphoric acid and distilled until about 12 ml. remained. CO₂-free distilled water (50 ml.) was then added to the distilling flask and distillation continued. This was repeated until all volatile acids were distilled over. The volatile acids were then determined according to Duclaux. The distillation constants were calculated from our own data obtained with pure acids.

Lactic acid was estimated directly on the fermented medium by the Fincke method (Official Methods, 1935).

Acetylmethylcarbinol was estimated on 200 ml. of the fermented medium according to Kluyver, Donker & Visser 't Hooft's (1923) modification of Lemoigne's method.

Fermentation of maize mash

5% maize mashes were fermented by the organism at 37° and 30°. The inoculant used was 1 ml. of a young active culture. The analyses of the products of fermentation are recorded in Tables 4 and 5.

Table 4. *Products of fermentation of 5% maize mashes at 37°*

Series I and II: 600 ml. fermented mashes after 6 days. Series III: 200 ml. fermented mash after 7 days.

	I	II	III
% starch fermented	82.79	64.22	48.69
Titrateable acidity (ml. 0.1 N acid/10 ml.)	6.89	7.40	6.25
	% on fermented starch		
Butyric acid	9.14	15.11	22.88
Acetic acid	9.49	11.33	11.99
Lactic acid	6.49	2.5	—
Formic acid	0.03	0.17	—
Acetone	5.44	1.30	0.19
<i>iso</i> Propanol	1.81	0.98	1.41
Butanol	9.42	3.12	0.0
Ethanol	5.02	7.17	6.23
Acetylmethylcarbinol	0.29	0.23	—
Total neutral solvents	21.98	12.80	7.83

Table 5. *Products of fermentation of 200 ml. 5% maize mash after 7 days at 30°*

	I	II
% starch fermented	61.85	53.37
Titrateable acidity (ml. 0.1 N acid/10 ml.)	8.8	8.3
	% on fermented starch	
Butyric acid	25.46	18.30
Acetic acid	13.35	23.71
Acetone	0.71	3.09
Butanol	3.00	6.04
Ethanol	11.88	6.15
<i>iso</i> Propanol	—	0.78
Total neutral solvents	15.59	16.06

The results of the analyses show that the main products of the fermentation of maize are butyric and acetic acid. It is evident, therefore, that the organism cannot be classified as a true butylic organism; more evidence on this matter is given below, in the discussion of its systematic relationships.

Butyric acid and acetic acids are also the chief products of dissimilation of glucose. Fair amounts of ethanol are produced, small amounts of butanol, and slight amounts of acetone, *isopropanol* and acetylmethylcarbinol. The organism produces more ethanol in the absence of calcium carbonate. Complete fermentation of the glucose takes place when the acids produced are neutralized.

In interpreting the above data for the identification of the new chromogen, it will be observed that the organism has many characteristics in common with both the two closely related specific groups: the butyric group represented by *Cl. butyricum* Prazmowski, and the so-called butylic group represented by *Cl. acetobutylicum* Weizmann, including the two known chromogens *Cl. felsineum* and *Cl. roseum*. The new organism resembles more closely the former group in morphology and in producing butyric acid rather than butanol; but it is a gelatin liquefier and none of the true butyrics is known to liquefy gelatin. Since the liquefaction of gelatin has been used so far as a diagnostic feature of the butyrics, one cannot lightly consider the new organism as a pigmented variety of *Cl. butyricum*, nor is there sufficient justification for regarding it as a pigmented variety of *Cl. acetobutylicum*.

COMPARISON WITH RELATED SPECIES

To facilitate the identification of the pink organism, it is compared with the closely related butylic chromogens *Cl. felsineum* and *Cl. roseum*.

The salient characteristics common to all three chromogens are briefly: (1) pinkish to reddish pigmentation on nutrient agar; (2) orange to reddish discoloration of maize and potato mashes; (3) liquefaction of gelatin; (4) the fermentation of pectin to a certain extent; (5) production of acetone, butanol, butyric acid and acetylmethylcarbinol.

The features that distinguish the new chromogen from *Cl. felsineum* and *Cl. roseum* are: (1) its partial diastatic action in maize mash, as against the complete diastatic activity of the butyrics; (2) its appreciably lower yields of neutral fermentation products from carbohydrates; (3) its inability to ferment inulin; (4) its failure to disintegrate potato tissue, whereas *Cl. felsineum* completely digests potato tissue to a yellow slime, producing abundant gas and a butyl odour, and *Cl. roseum* behaves similarly to *Cl. felsineum* but forms a clear yellow liquid and bluish sediment; (5) its inability to reduce nitrates; (6) its optimum temperature of growth at 30° as compared with 37° for *Cl. felsineum* and *Cl. roseum*; (7) certain appreciable differences in pigmentation.

A closer comparison of the individual fermentation products provides further evidence of a difference between the three organisms. In Tables 6 and 7, the yields of the major fermentation products obtained by the new organism W45 in a number of experiments are compared with those recorded for *Cl. roseum* and *Cl. felsineum* (Kluyver strain). The fact that W45 is a poor solvent producer from maize mash is clearly shown.

The production of *isopropanol* and acetylmethylcarbinol is noteworthy. W45 produces *isopropanol* from carbohydrates, whereas, according to McCoy and co-workers, *Cl. roseum* and *Cl. felsineum* do not. Van der Lek (1930),

also working with the Kluyver strain of *Cl. felsineum*, found isopropanol in a few instances only. McCoy and co-workers do not record the production of acetylmethylcarbinol by *Cl. felsineum* and *Cl. roseum*; Van der Lek, however, observed its production from glucose by *Cl. felsineum*. The new chromogen forms it too. Besides the results recorded in Tables 3 and 4, three confirmatory

Table 6. *Fermentation of 5% maize by W 45, Cl. roseum* and Cl. felsineum* at 37°*

	W 45					<i>Cl. roseum</i>	<i>Cl. felsineum</i>
	1	2	3	4	5		
Total solvents (g./l.)	1.88	2.48	1.58	1.44	2.19	11.7	12.2
Composition of neutral solvents as % of total solvents							
Acetone	2.17	7.07	11.75	3.48	0.00	27.6	30.5
Butanol	32.61	27.27	43.49	21.25	58.81	59.9	60.4
Ethanol	65.22	65.66	44.76	75.26	41.19	12.6	9.0
Final acidity (ml. 0.1N acid/10 ml.)	7.1	7.9	7.9	7.3	5.9	3.4	2.3
Residual starch (iodine test)	Present	Present	Present	Present	Present	Absent	Absent

* Figures for *Cl. roseum* and *Cl. felsineum* taken from Langlykke *et al.* (1935).

Table 7. *Fermentation of yeast-glucose by W 45, Cl. roseum* and Cl. felsineum* at 37°*

	W 45		<i>Cl. roseum</i> strain 42	<i>Cl. roseum</i> strain 43	<i>Cl. felsineum</i> strain 41
	1	2			
Glucose fermented	60.4 %	45.9 %	56.5 %	46.5 %	79.6 %
Acidity (ml. 0.1N acid/10 ml.)	6.4	6.9	9.85	9.05	6.70
Neutral volatile products, as % of glucose fermented					
Butanol	4.9	3.5	4.6	2.6	14.4
Ethanol	8.7	11.7	3.5	3.1	1.8
Acetone	1.0	0.6	1.5	1.0	4.4
isoPropanol	1.8	2.1	—	—	—
Total solvents	16.4	17.9	9.6	6.9	20.6

* Figures for *Cl. roseum* and *Cl. felsineum* taken from Langlykke *et al.* (1935).

flask experiments were made with 100 ml. of a 2% glucose nutrient medium. Two of the flasks containing calcium carbonate gave 3.4 and 6.5 mg. and the third flask without calcium carbonate gave 3.2 mg. of acetylmethylcarbinol.

Action on pectin. The new chromogen definitely ferments pectin (see Table 2), a characteristic shared by *Cl. roseum* and *Cl. felsineum*. When grown on carrot wedges, the organism causes a marked disintegration of the tissue (Pl. 1 C 2). Definite signs of softening and rotting are observed within 48 hr.

Microscopic examination of the softened tissues reveals that a certain amount of separation of the cells has occurred, but that the cell walls are left intact. On staining with ruthenium red, the stain is in many instances localized in certain places and not evenly distributed around the cell walls. This suggests that the pectic substances of the middle lamella cementing the cells together

are attacked by the organism. Potato slopes on the other hand show no signs of disintegration (Pl. 1, C1). It is interesting to compare this with certain inoculation experiments carried out on potato and carrot slopes with strains of *Cl. pectinovorum* (Weizmann & Hellinger, 1940). Two of the strains brought about a slow but perceptible breakdown of the potato tissue, but the carrot slope was left intact.

The ability of the new bacterium to attack pectin is of interest because of the claims made by various investigators that certain species of clostridia are the causal agents of retting; *Cl. felsineum* is known to be a true retting organism.

Table 8. *Comparative measurements of W45, Cl. roseum and Cl. felsineum*

	W45 24 hr. on maize (μ .)	<i>Cl. roseum</i> 24 hr. on maize* (μ .)	<i>Cl. felsineum</i> † (μ .)
Rods:			
Limits of size	2.6–13.0 $\times$ 0.5–0.6	3.2–4.3 $\times$ 0.7–0.9	3.0–5.0 $\times$ 0.3–0.4
Average	5.6 $\times$ 0.54	3.9 $\times$ 0.8	
Clostridia:			
Limits of size	4.5–8.0 $\times$ 0.7–1.0	5.3–8.8 $\times$ 1.1–1.5	—
Average	5.8 $\times$ 0.82	6.7 $\times$ 1.4	
Spores:			
Limits of size	2.0–2.4 $\times$ 0.5–1.1	2.5–2.8 $\times$ 1.2–1.3	2.3 $\times$ 1.5–2
Average	2.2 $\times$ 0.95	2.7 $\times$ 1.2	
Spore position	Sub-terminal	Sub-terminal	Sub-terminal

* McCoy & McClung's (1935) measurements.

† Carbone & Tomolato's (1917) measurements.

Pigmentation. The new clostridium generally produces orange-red colonies on yeast glucose agar, but sometimes they may be faintly pink and deepen on standing under anaerobic conditions to a pale orange. The colour is more intense at the centre of the colony, where growth is thicker. On first isolation colonies showed the most intense pigmentation. After numerous subculturings, the colour was less and appeared irregularly. Some colonies develop pigmentation only after some weeks. Pigment production, however, always occurs on maize; on exposure to air, the colour fades in maize mash. Intermittent sub-cultivation from maize mash on to yeast glucose agar appears to intensify the pigmentation.

Colonies of *Cl. roseum* on beef peptone glucose agar are stated by McCoy & McClung (1935) to be orange-red; the colour darkens, on exposure to air to almost black, and a purplish pigment diffuses into the agar for several millimeters from the colonies. According to Ruschmann & Bavendamm (1925), colonies of *Cl. felsineum* on carrot juice agar are yellow-red, and the colour darkens on standing in the air to a dirty grey-brown and finally blackish brown. A purplish colour is said to develop from the surface downward in maize cultures of *Cl. roseum* left standing in the air.

Morphology. Too much reliance should not be placed on the value of a comparison of the morphological data summarized in Table 8. The rods of *Cl. roseum* and *Cl. felsineum* appear to be more regular than W45, whereas

W45 appears to be thinner than *Cl. roseum*. Spores of W45 are smaller than the spores of *Cl. roseum* and *Cl. felsineum*. Ruschmann & Bavendamm (1925) observed that Carbone & Tomolato's *Cl. felsineum* produced, on potato mash, long threads which broke up into cell-chains of many segments, and also double and single rods. This characteristic has frequently been observed with W45 on various media.

Sporulation. When subcultured several times at 37°, the new organism loses its ability to sporulate, but quickly recovers it at 30°. This feature does not appear to be a characteristic of *Cl. felsineum* or *Cl. roseum*.

Identity of the organism

There is a certain resemblance between the new chromogen and the two butyl chromogens *Cl. felsineum* and *Cl. roseum*, but it is outweighed by the differences. The new bacterium is a butyric organism, but it is unusual in that it possesses a gelatinase. The organism is, therefore, recognized as a new *Clostridium* for which the name *Cl. aurantibutyricum* is suggested.

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A. Colony of *Cl. aurantibutyricum*. 48 hr. growth on yeast glucose agar under anaerobic conditions at 30° ($\times 23$).

B. Clostridial forms from a 6 days old colony of *Cl. aurantibutyricum* on yeast glucose agar at 30° ($\times 1440$).

- C. 1. Potato wedge, inoculated with *Cl. aurantibutyricum*, scarcely attacked after 21 days.
 2. Inoculated carrot wedge which has disintegrated and fallen into the aqueous solution below the constriction of the tube.

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