Characterization of Strains of *Clostridium puniceum* sp.nov., a Pink-pigmented, Pectolytic Bacterium

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The properties of ten strains of pink-pigmented, pectolytic clostridia implicated in spoilage of potatoes and one strain isolated from carrots have been compared with reference strains. On the basis of the position of spore formation, ability to degrade gelatin, and formation of butanol plus acetic and butyric acids, the organisms resemble *Clostridium acetobutylicum*, *C. aurantibutyricum* and *C. felsineum*, but they differ sufficiently from these bacteria to warrant recognition as a new species.

INTRODUCTION

Pectolytic clostridia of several morphological types can be isolated from decaying vegetable material, particularly from potato tubers (Veldkamp, 1970; Lund, 1972), and probably contribute to spoilage of potatoes (Lund & Kelman, 1977; Lund, 1979; Pérombelon *et al.*, 1979). Strains of pectolytic clostridia also degrade carrot tissue and have been claimed to be a cause of cavity spot (Perry & Harrison, 1977, 1979).

Some strains of pectolytic clostridia form a pink, cell-bound pigment; a similarly pigmented clostridium was reported to enhance rotting of potatoes by *Erwinia carotovora* var. *atroseptica* (Rudd-Jones & Dowson, 1950) but was not characterized. This paper describes some properties of 11 newly isolated strains of pink-pigmented, pectolytic clostridia compared with reference species. It is concluded that the isolates should be regarded as strains of a new species.

METHODS

Bacteria. Cultures are listed in Table 1. Ten of the 11 strains studied were isolated from rotting potatoes in seven separate experiments. Eight of these strains were isolated in Norwich, and two (BL75/100 and BL75/129) in Madison, Wisconsin, U.S.A.; eight strains were from tubers rotting at 20 °C while two strains (BL72/60 and BL72/61) were from tubers rotting at 10 °C. One strain (BL78/21) was obtained from Dr D. A. Perry, Scottish Horticultural Research Institute, Dundee, and was isolated from a cavity spot lesion of carrot. Reference cultures were from the National Collection of Industrial Bacteria (NCIB).

Anaerobic techniques. For routine culture and for many biochemical tests, the techniques used to maintain anaerobiosis were those described by Willis (1969). In tests which involved prolonged incubation or the use of minimal media, anaerobiosis was maintained by a modification of the strictly anaerobic techniques described by Holdeman *et al.* (1977).

Media for isolation, maintenance and inoculation of biochemical tests. The pectolytic clostridia were isolated on a pectate medium containing polymyxin (PI-PMS; Lund, 1972) and purified by repeated plating on the same medium and on Reinforced Clostridial Medium (RCM; Hirsch & Grinstead, 1954) or Potato Infusion Agar (PIA) which contained (l⁻¹): potato infusion, 500 ml; glucose, 5 g; (NH₄)₂SO₄, 1 g; CaCO₃, 3 g; cysteine.HCl, 0.5 g; Bacto-agar (Difco), 15 g. All plates were incubated under H₂/CO₂ (9:1, v/v) for 16 h before use, and after inoculation. Cultures were maintained in Potato Infusion Medium (PIM; Conn, 1957) to which was added cysteine.HCl (0.5 g l⁻¹). Subcultures for inoculating media for biochemical tests were grown in VL medium (Barnes & Impey, 1968). Before use, bottles of liquid medium were held in a boiling water bath for 20 min to remove dissolved oxygen, cooled and immediately inoculated. Cultures were incubated at 25 °C unless specified otherwise.

Morphology. Descriptions of colony morphology were based on cultures grown on PIA under H_2/CO_2 (9:1, v/v) for 4 d. Cultures grown in VL medium or in RCM for 8 h at 25 °C, or at 37 °C in the case of *C. felsineum*, were used for Gram stains [Jensen's modification (Cruickshank, 1965) decolorizing with acetone or ethanol] and for measurements of vegetative bacteria. *Streptococcus faecalis* and *Escherichia coli* were used as controls for the Gram stain. Measurements of vegetative bacteria were made using smears stained with Loeffler's methylene blue and an eyepiece micrometer calibrated against a stage micrometer graduated in 10 μm divisions. Spores, formed after growth on PIA for up to 1 month at 25 °C, were stained by Schaeffer & Fulton's method (Cowan, 1974) and measured using an eyepiece micrometer. Bacteria grown in VL medium for 4 d or on PIA for 7 d were examined for capsules by the wet-mount method (Duguid, 1951). The presence of granules in clostridial forms was detected by staining with Lugol's iodine solution (McCoy *et al.*, 1926).

For electron microscopy, cultures were grown on PIA for 24 h, in the case of vegetative cells, or for up to 30 d when maximum sporulation was required. Colonies of vegetative cells were picked from the medium, gently emulsified in distilled water and a drop of suspension was immediately transferred to a carbon-coated grid (400 mesh) and stained with a saturated, aqueous solution of uranyl acetate. Spores were washed from the medium with sterile distilled water, sedimented by centrifugation (30 000 g, 20 min, 3 °C), washed five times with potassium phosphate buffer (50 mM, pH 7.0), resuspended in distilled water and negatively stained as described above. The preparations were examined in an AEI 801 electron microscope operating at 60 kV.

Pigment. Cultures were grown on PIA containing skim milk powder (50 g l⁻¹). After incubation for 12 d, colonies were scraped from the medium and the pigment was extracted with hot ethanol. The solution was concentrated on a rotary evaporator at 40 °C and examined spectrophotometrically.

Optimum growth temperature and temperature limits for growth. A thermal-gradient incubator was used, similar in principle to that described by Matches & Liston (1973). An aluminium block contained four rows of 20 tubes and was used with a temperature gradient of 0 to 44 °C between the extreme ends of the block. The temperature was monitored regularly by means of 20 thermistors positioned in the block. The range of temperature variation in each tube was between ± 1.3 °C at 10 °C and ± 0.9 °C at 40 °C. Using strictly anaerobic techniques, tubes containing 10 ml medium A (described below) were allowed to equilibrate in the block, and inoculated with 0.5 ml of a 2 or 3 d culture in VL medium. Growth was measured by increase in turbidity using an EEL nephelometer standardized to give a reading of 100 with the reference turbidity tube. The range of temperature allowing growth was determined from the tubes showing a turbidity of more than 30 after 28 d, and the results are reported to the nearest 0.5 °C.

The ability of strains to grow at 10 and 15 °C was also tested by inoculating 20 ml VL medium with 1.5 ml of a culture grown in VL medium for 3 d at 25 °C, and incubating for up to 30 d.

Requirement for fermentable carbohydrate. Growth of bacteria was compared in medium A, which contained glucose (10 g l⁻¹), and in medium B, in which glucose was replaced by Oxoid casein hydrolysate (acid) (18 g l⁻¹). The base for media A and B contained salts solution (Holdeman *et al.*, 1977) to which was added (l⁻¹): adenine, guanine, uracil, vitamin B₁₂, each 100 μg ; thiamin, riboflavin, each 500 μg ; pyridoxal.HCl, folic acid, each 250 μg ; nicotinic acid, 5 mg; calcium pantothenate, 1 mg; biotin, *p*-aminobenzoic acid, each 50 μg ; inositol, 16 mg; choline chloride, 200 mg (Mead, 1971); (NH₄)₂SO₄, 1.0 g; Oxoid casein hydrolysate (acid), 2 g; tryptophan, 0.4 g; cysteine.HCl, 0.5 g; resazurin, 1 mg; NaHCO₃, 5 g. A solution of the vitamins at 100 times the final concentration was sterilized by filtration through a cellulose acetate filter (0.22 μm pore size, Millipore). The media components, apart from vitamins, cysteine.HCl and NaHCO₃, were dissolved in 900 ml distilled water, the pH was adjusted to 6.8 and the solution was autoclaved under a headspace of CO₂ using the techniques described by Holdeman *et al.* (1977). Cysteine.HCl and NaHCO₃ were each separately dissolved in 50 ml distilled water and autoclaved under a headspace of CO₂ or N₂, respectively. After sterilization the solutions of cysteine.HCl and NaHCO₃ and 10 ml vitamin solution were added to the remaining 900 ml medium and 10 ml amounts were dispensed into tubes using strictly anaerobic techniques. Cultures were incubated for up to 21 d and growth was measured by increase in turbidity using an EEL nephelometer.

Disintegration of raw potato. Cylinders of raw potato tissue prepared aseptically and immersed in a sterile solution of cysteine.HCl (0.5 g l⁻¹) (Lund & Brocklehurst, 1978) were inoculated and incubated for up to 14 d. An equal number of uninoculated, control cylinders of tissue showed no interfering contamination.

Degradation of pectate. Ability to degrade pectate was detected by the formation of craters surrounding colonies on PI-PMS medium within 7 d.

Degradation of gelatin. Four methods were used: (1) charcoal-gelatin discs (Oxoid) in VL medium; (2) a nutrient medium containing glucose (1 g l⁻¹) and gelatin (150 g l⁻¹) (Barnes *et al.*, 1966); (3) the method of Holdeman *et al.* (1977); (4) gelatin agar plates (Frazier, 1926) modified by increasing the glucose concentration to 2.5 g l⁻¹ (McCoy & McClung, 1935). The tests were incubated at 25 °C for 28 d in methods 1, 2 and 3, and for 8 d in method 4. In methods 2 and 3 a positive result was recorded if the medium remained liquid after cooling to +1 °C.

Degradation of casein. The medium contained (g l^{-1}): skim milk, 50; cysteine.HCl, 0.4; agar (Davis), 15; pH 7.2 to 7.4. Tests were incubated for 8 d. Degradation of casein was shown by formation of a clear zone which did not become opaque when flooded with 0.1 M-HCl (McCoy *et al.*, 1926).

Degradation of starch, carboxymethylcellulose and tributyrin. The basal medium contained (g l^{-1}): Tryptone (Oxoid), 10; beef extract powder (Lab Lemco), 2.4; yeast extract (Difco), 5; cysteine.HCl, 0.4; agar (Davis), 15; pH 7.2 to 7.4. Ability to degrade starch was tested by incorporating potato starch (BDH; 10 g l^{-1}) into the basal medium; the development of an opaque zone around colonies indicated degradation of the substrate (Iverson & Millis, 1974) and was confirmed by flooding with a 1 in 5 dilution of Lugol's iodine solution. Ability to degrade carboxymethylcellulose (Hercules Inc., Wilmington, Del., U.S.A.) was tested by incorporating the substrate (10 g l^{-1}) and glucose (1 g l^{-1}) into the basal medium; breakdown of the polymer was shown by the presence of a clear zone around colonies after flooding with a 1% (w/v) aqueous solution of hexadecyltrimethylammonium bromide (Hankin & Anagnostakis, 1977). To test for degradation of tributyrin, triple-layer plates were used; the bottom and top layers contained basal medium plus glucose (1 g l^{-1}), while the middle layer consisted of the same medium plus glyceryltributyrate (30 ml l^{-1}) (Barnes & Impey, 1972). Breakdown of tributyrin resulted in a clear zone around colonies. In each case, tests were incubated for 8 d.

Utilization of carbohydrates. The methods of Holdeman & Moore (1972) were used; media were inoculated with approximately 0.1 ml of a vigorous culture in VL broth, under a stream of oxygen-free nitrogen to exclude air. Galactose, glycerol, glycogen (oyster) and soluble starch were obtained from BDH; the remaining substrates were from Sigma. Tests were incubated for 8 d and utilization of carbohydrates was indicated by growth and a decrease of pH from between 6.5 and 7.0 to 6.0 or less.

Coagulation of skim milk. The method of Barnes & Impey (1968) was used, with incubation for 14 d.

Production of hydrogen sulphide. Ferrous sulphate and sodium thiosulphate solutions were separately sterilized and added to BGP medium (Barnes & Impey, 1968) to give concentrations of 0.2 and 0.3 g l^{-1} , respectively (Beerens & Tahon-Castel, 1965). Production of H_2S was indicated by blackening of the cells within 7 d.

Reduction of nitrate. Glucose and sodium nitrate solutions were separately sterilized and added to basal medium (Barnes & Impey, 1968) to give concentrations of 2 and 5 g l^{-1} , respectively. After incubation of cultures for 7 d reduction of nitrate was detected by the methods of Cowan (1974).

Formation of lecithinase. Cultures were grown on egg yolk agar (Holdeman *et al.*, 1977) containing egg yolk emulsion (Oxoid, 50 ml l^{-1}) for 7 d.

Formation of indole. Cultures were grown in 20 ml VL medium for 7 d; 1 ml Kovacs' reagent (Cowan, 1974) was added to detect indole.

Fermentation products from glucose. Cultures were grown in VL medium for 7 d. After acidifying the cultures, volatile fatty acids and alcohols were extracted with ether (Holdeman *et al.*, 1977) and detected using a gas-liquid chromatograph (Pye Unicam model 104) with a glass column ($1.5 \text{ m} \times 0.4 \text{ mm i.d.}$) packed with 20% PEG 20M on 2% H_3PO_4 -treated 100/120 mesh Diatomite C (Pye Unicam) and dual flame-ionization detectors. The column temperature was 120°C for alcohols and 150°C for methylated fatty acids. Flow rates to detectors were: carrier gas (argon), 50 ml min^{-1} ; hydrogen, 50 ml min^{-1} ; air, 750 ml min^{-1} .

A more quantitative assay of the volatile fatty acids, butanol and acetone formed was made for some representative strains. Cultures were grown for 7 d at 25°C in medium containing (g l^{-1}): peptone (Evans), 7.5; beef extract powder (Lab-Lemco), 4; yeast extract (Oxoid), 3; K_2HPO_4 , 4; glucose, 5; cysteine.HCl, 0.5; NaHCO_3 , 0.2 (sterilized separately as a solution containing 20 g l^{-1}); pH 7.2 to 7.4. After separating the cells by centrifugation, residual glucose was estimated by the glucose oxidase method (Biochemica Test Combination, Boehringer). Volatile fatty acids were separated by the steam distillation method of Annison (1954) and assayed by gas-liquid chromatography as described by Barnes *et al.* (1977). The concentrations of individual acids (except formic, to which the detectors are insensitive) were determined from their peak areas relative to that of the internal standard, corrected by previously determined detector response factors. To assay acetone and butanol, samples ($1 \mu\text{l}$) of culture supernatant liquid were injected directly into the gas-liquid chromatograph, using column and detector temperatures of 85°C and 100°C , respectively. Acetone and butanol concentrations were determined by reference to a calibration plot of peak area against concentration for aqueous solutions containing a mixture of known weights of these compounds. Linear responses were obtained for acetone over the range 10 to $400 \mu\text{g ml}^{-1}$ and for butanol from 50 to $1500 \mu\text{g ml}^{-1}$.

Determination of DNA base ratio. For growth of cells, the culture medium, either VL or RCM, was sterilized in 2.5 l volumes in 3 l Erlenmeyer flasks. Stoppers with inlet and outlet tubes were sterilized separately and fitted to the flasks immediately after autoclaving. During cooling a slow stream of H_2/CO_2 (9:1, v/v) was passed through the medium via a catalytic gas purifier (Deoxo Purifier model D; Engelhard Sales, Valley Road, Cinderford, Glos.). The medium was inoculated with 60 ml of a culture in VL medium, the gas inlet tube was clamped off and a Bunsen valve was attached to the outlet tube to allow escape of gases formed during growth. The flasks were incubated at 25°C for the potato isolates, and at 30°C for *C. felsineum* and *C. aurantibutyricum*,

until growth had reached the exponential phase as indicated by increase in turbidity and rate of evolution of gas (1 to 3 d). Cultures were then harvested by centrifugation and washed twice with saline/EDTA solution (NaCl, 0.15 M; EDTA, 0.15 M; pH 8.0).

Cells of the potato isolates and *C. aurantibutyricum* were disrupted by suspending 3 to 5 g wet wt in saline/EDTA solution in the presence of 10 mg lysozyme and incubating at 37 °C for 1 h. Cells of *C. felsineum* were disrupted in a Hughes press. In each case, lysis was completed by heating at 60 °C for 15 min in saline/EDTA solution containing 3% (w/v) sodium dodecyl sulphate. Further isolation of DNA and determination of the mol% GC content by the thermal denaturation method followed the procedures of Marmur (1961) and Marmur & Doty (1962) with modifications described by Barnes *et al.* (1977).

RESULTS

The pink-pigmented clostridia were isolated on media incubated under H₂/CO₂ (9:1, v/v) and failed to grow on media incubated in air.

Morphology and pigment

When grown in VL medium for 7 to 8 h at 25 °C the isolates were rods (0.6 × 1.8 to 4.2 µm) which were predominantly Gram-negative. The bacteria were motile with peritrichous flagella (Fig. 1e) and capsules were not detected. On PIA medium under H₂/CO₂ (9:1, v/v) the vegetative bacteria developed into motile clostridial forms containing granulose and in which oval spores were subsequently formed in a subterminal position (Fig. 1a to d). The free spores measured 0.9 to 1.2 × 1.5 to 2.4 µm and were surrounded by an extensive exosporium (Fig. 1f) with subunits showing an hexagonal periodicity similar to that described in detail for strain BL70/20 (Lund *et al.*, 1978).

After growth on PIA under H₂/CO₂ (9:1, v/v) for 4 d at 25 °C, colonies were circular or circular/irregular, with a maximum diameter of 1 to 2.5 mm, usually convex (one strain low convex, two strains convex-umbonate), amorphous, smooth, undifferentiated or differentiated into central and peripheral regions, edge entire, or entire-undulate or entire-lobate, pale pink to deep pink, opaque, viscid and difficult to emulsify. Colonies of group A strains (Table 1) became mucoid after longer incubation. Despite the viscid and mucoid nature of colonies, capsules were not detected on individual cells from PIA.

On PIA the pigment was mainly confined to the colonies and appeared similar to that of *C. rubrum*. Pigment extracted from representative strains (BL70/20, BL72/60, BL72/61, BL73/46 and BL74/100) showed an absorption maximum, in ethanol, at 542 nm; pigment from *C. rubrum* strains NCIB 9503 and NCIB 9504 showed similar spectra to the five previous strains but with an absorption maximum at approximately 546 nm.

Requirement for a fermentable carbohydrate

Tests of strains BL70/20, BL72/60, BL72/61 and BL73/46 showed that no growth occurred in 21 d in medium B (without fermentable carbohydrate), whereas in medium A (containing glucose, 10 g l⁻¹) heavy growth had occurred after 4 d.

Degradation of gelatin

Tests by methods 1, 2 and 3 failed to give clear positive results indicating breakdown by the isolates or by reference cultures of *C. felsineum*. Method 2 showed evidence of partial breakdown resulting in a lowering of the temperature required to solidify the gelatin medium; however, the cultures solidified at +1 °C and the results of the test were therefore recorded as negative. The ability of the isolates and the reference cultures of *C. felsineum* to degrade gelatin was clearly demonstrated by method 4, whereas reference cultures of *C. butyricum* and *C. rubrum* gave negative results. The advantage of using the sensitive gelatin agar plate method was discussed by Willis & Williams (1970).

Fig. 1. Morphology of strain BL70/20.

(a–d) Phase contrast photomicrographs of cells grown on PIA at 25 °C for 1, 2, 3 and 4 d, respectively: (a) vegetative cells; (b) clostridial forms; (c) sporulating cells; (d) free spores. Bar markers represent 5 μ m.

(e–f) Electron micrographs of negatively stained cells: (e) vegetative cells grown as in (a); (f) free spores grown as in (d). Bar markers represent 1 μ m.

Comparison of pink-pigmented, pectolytic strains of clostridia with reference *Clostridium* spp. of groups I and II (Smith & Hobbs, 1974)

Properties of the isolates and of reference strains are shown in Table 1. In view of the subterminal position of spore formation the isolates were comparable with *Clostridium* spp.

Table 1. *Comparison of properties of pink-pigmented, pectolytic strains of Clostridium and of reference strains*

Group A strains were BL70/20, BL75/100, BL75/129, BL76/37, BL76/38 and BL78/21; group B strains were BL72/60, BL72/61, BL73/46, BL74/100 and BL74/101; *C. felsineum* strains were NCIB 10690, NCIB 9539 and NCIB 9540; *C. rubrum* strains were NCIB 9503 and NCIB 9504.

Character	Group A (6 strains)	Group B (5 strains)	<i>C. felsineum</i> (3 strains)	<i>C. aurantibutyricum</i> NCIB 10659	<i>C. acetobutylicum</i> NCIB 8052	<i>C. butyricum</i> NCIB 7423	<i>C. rubrum</i> (2 strains)
Gram stain	—	—	+ (+)*	+ (+)	+ (+)	NT	NT
Exosporium	+	+	— (2/3) (—)†	+	+ (—)	NT	+—
Pigment	Pink	Pink	Yellow/brown	White	White	White	Pink
Temperature range for growth (°C)	13–37.5‡	7–39§	19–41	14–43.5	NT	NT	NT
Optimum temperature (°C)	28.5–33‡	23–30§	30–39	32–41	NT	NT	NT
Growth at 10 °C	—	+	—	—	NT	NT	NT
Growth at 15 °C	+	+	—	+	NT	NT	NT
Degradation of:							
Potato	+	+	+	+	—	—	—
Pectate	+	+	+	+	—	—	—
Gelatin	+	+	+ (+)	+ (+)	+ (+)	— (—)	— (—)
Casein	+	— (4/5)	+ (2/3) (—)	— (—)	— (d)	—	—
Tributyrin	—	—	—	+	—	NT	NT
Coagulation of skim milk	+	+	+	+	+	NT	—
Fermentation products from glucose	BA4	BA4	BA4	BA4	NT	NT	BA
Fermentation of:							
Amygdalin	+	+	— (d)	— (—)	— (d)	— (d)	— (+)
Glycogen	— (5/6)	+	— (d)	+ (+)	+ (+)	+ (+)	+ (+)
Inositol	—	—	— (—)	— (—)	+ (d)	+ (d)	— (—)
Inulin	—	+	— (d)	— (—)	— (d)	— (d)	+ (+)
Lactose	+	+	— (+)	+ (+)	+ (+)	+ (+)	+ (+)
Maltose	+	+	— (d)	+ (+)	+ (+)	+ (+)	+ (+)
Mannitol	—	+	— (—)	— (—)	— (d)	— (d)	— (d)
Melezitose	—	+	— (—)	— (—)	— (—)	— (—)	— (+)
Melibiose	+	+	— (—)	+ (—)	— (—)	— (+)	— (—)
Raffinose	+	—	— (d)	+ (+)	— (d)	+ (+)	+ (—)
Rhamnose	—	—	+ (+)	— (—)	— (d)	— (d)	— (d)
Ribose	—	+	— (—)	+ (—)	— (—)	— (+)	— (d)
Trehalose	+	+	— (—)	— (—)	+ (+)	+ (+)	+ (d)
Formation of acetylmethyl-carbinol	—	—	+ (+)	— (—)	— (+)	—	—
Reduction of nitrate	—	—	— (—)	+ (+)	— (—)	—	—
GC content of DNA (mol %)	28–29‡	28–29§	28–29	27–28	NT	NT	NT

All cultures degraded potato starch, none attacked carboxymethylcellulose or lecithin; all utilized arabinose, cellobiose, fructose, galactose, glucose, mannose, salicin, sucrose and xylose, but failed to utilize dulcitol, glycerol, sorbitol or sorbose; all hydrolysed aesculin, none formed indole or hydrogen sulphide.

+, >90% strains positive; —, >90% strains negative; d, 11 to 89% strains positive; NT, not tested.

* Results in parenthesis are those given by Smith & Hobbs (1974).

† Where differences occurred between strains the proportion giving the majority result is shown in parenthesis.

‡ Determined for strain BL70/20.

§ Determined for strains BL72/60, BL72/61 and BL73/46.

|| B, Butyric acid; A, acetic acid; 4, butanol (Holdeman *et al.*, 1977).

Table 2. Assay of volatile fatty acids and neutral solvents formed by pink-pigmented, pectolytic strains of *Clostridium* and of *C. rubrum*

The growth medium contained (g l⁻¹): glucose, 5; peptone, 7.5; beef extract powder, 4; yeast extract, 3; K₂HPO₄, 4; cysteine.HCl, 0.5; NaHCO₃, 0.2; pH 7.2 to 7.4. The amounts of products formed are expressed as μ mol per 100 μ mol glucose fermented.

	Strain			
	BL70/20	BL72/61	BL73/46	<i>C. rubrum</i> NCIB 9504
Glucose fermented (%)	100	100	100	100
Acetone	7.9	<0.7	<0.7	<0.7
Butanol	51.7	65.5	26.9	<0.7
Acetic acid	30.4	30.4	22.4	16.6
Butyric acid	15.2	17.2	34.5	59.7

of groups I and II (Smith & Hobbs, 1974). The ability of the 11 strains to degrade gelatin differentiated them from group I species, including *C. butyricum* and *C. rubrum*.

The 11 strains could be divided into two groups, A and B. Group A, in contrast to group B, failed to grow at 10 °C, to ferment inulin, mannitol, melezitose or ribose but fermented raffinose; only one group A strain fermented glycogen.

Like the group II species *C. acetobutylicum*, *C. felsineum* and *C. aurantibutyricum*, the isolates formed substantial amounts of butanol which accounted for up to 43% (w/w) of the glucose carbon (Table 2). They differed from *C. acetobutylicum* in colony characteristics, pigment, ability to macerate potato and degrade pectate, and there were minor differences in the sugars fermented. The isolates differed from *C. felsineum* and *C. aurantibutyricum* in being predominantly Gram-negative in the exponential phase of growth, although the granulose-containing clostridial forms were Gram-positive. They differed from *C. felsineum* in colony morphology, pigment, ability to ferment several carbohydrates and failure to form acetylmethylcarbinol. Exosporia were not detected on spores of *C. felsineum* NCIB 10690 or NCIB 9539, but spores of strain NCIB 9540 showed a close-fitting exosporium with a similar hexagonal periodicity to that present on the pink isolates. Group A and group B strains differed from *C. aurantibutyricum* in colony morphology, pigment, failure to attack tributyrin or reduce nitrate and ability to ferment amygdalin and trehalose; spores of *C. aurantibutyricum* possessed extensive exosporia which failed to show hexagonally arranged subunits.

DISCUSSION

The pigment formed by the 11 strains of pigmented, pectolytic clostridia resembled that formed by *C. rubrum*. Although the original isolates of this species were described as pectinolytic, they were only reported to ferment up to 6% (w/w) of pectate or pectin in the medium while other non-pigmented strains, similar to *C. multifementans* or to *C. butyricum*, fermented up to 80% (w/w) of these substrates (Ng & Vaughn, 1963). Whereas our isolates readily liquefied the pectate medium and macerated potato tissue, the reference strains of *C. rubrum* failed to do so; our strains also differed from *C. rubrum* in their ability to degrade gelatin, to coagulate skim milk and to form butanol.

Our isolates were similar in several respects to *C. acetobutylicum*, *C. felsineum* and *C. aurantibutyricum* but showed distinct differences from each of these species; the most useful characteristics differentiating these bacteria are shown in Table 3. In addition to the differences reported here between the pink-pigmented strains and *C. felsineum*, previous work showed that five representative isolates and *C. aurantibutyricum*, when grown in potato

Table 3. *Characteristics most useful in differentiating the pink-pigmented, pectolytic strains from reference species of Clostridium**

Character	Pink-pigmented, pectolytic strains	<i>C. felsineum</i>	<i>C. auranti- butyricum</i>	<i>C. aceto- butylicum</i>
Pigment on PIA	Pink	Orange/brown	White	White
Colony morphology on PIA	Circular- circular/ irregular Viscid	Irregular	Circular/ irregular	Circular
Degradation of:		Butyrous	Butyrous	Butyrous
Potato	+	+	+	-
Pectate	+	+	+	-
Tributyryn	-	-	+	-
Fermentation of:				
Amygdalin	+	- (d)	- (-)	- (d)
Melibiose	+	- (-)	+	- (-)
Rhamnose	-	+	- (-)	- (d)
Trehalose	+	- (-)	- (-)	+

+, >90% strains positive; -, >90% strains negative; d, 11 to 89% strains positive.

* This table includes our own results and, in parenthesis, those from Smith & Hobbs (1974).

tissue, formed pectate lyase and pectinesterase but no detectable polygalacturonase, whereas strains of *C. felsineum* formed pectate lyase and polygalacturonase but no detectable pectinesterase (Lund & Brocklehurst, 1978).

The reference strain of *C. aurantibutyricum* differed in two respects from the original description (Hellinger, 1947, 1952). It failed to form pigment, a fact also noted by Cummins & Johnson (1971), whereas the original isolate formed a pink to reddish colour on nutrient agar and an orange to red pigment on maize or potato mash; the reference strain also rapidly degraded potato tissue whereas the original strain failed to do so despite its reported ability to utilize pectin (Hellinger, 1947; Raynaud, 1949). Although our isolates showed many properties in common with the reference strain of *C. aurantibutyricum*, because of the differences found in this work and the differences between the reference strain and the original description by Hellinger, we cannot conclude that our isolates should be classified as *C. aurantibutyricum*.

In addition to *C. rubrum* and *C. aurantibutyricum*, the pink- or red-pigmented species described in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Smith & Hobbs, 1974), several species were described in the previous edition (Breed *et al.*, 1975; Raynaud, 1949), namely *C. venturellii*, *C. saturnirubrum*, *C. roseum*, *C. chromogenes*, *C. corallinum* and *C. carbonei*. Of these species only a culture of *C. roseum* was available. A major difference between *C. roseum* and *C. felsineum* was reported to be darkening of the pigment of *C. roseum* on exposure to air (McCoy & McClung, 1935); this species is now regarded as *C. felsineum* (G. Hobbs, personal communication). The descriptions of the remaining species either indicate significant differences from our isolates or are inadequate for a comparison to be made (refer to discussion by Ng & Vaughn, 1963).

The 11 strains described in this paper therefore differ from previously described clostridia. They all have a distinctive combination of properties, namely, formation of a similar pink pigment, ability to cause soft rots of potato and to degrade pectate, formation of subterminal spores with a similar morphology, ability to degrade gelatin and to form butyric and acetic acids and butanol from glucose. Although there are differences between the range of carbohydrates used by group A and group B strains, in view of the properties common to both groups the differences are not considered a sufficient reason to differentiate two species. Accordingly, a new species is proposed to include all 11 strains.

The following description is based on the type strain, BL70/20, which has been deposited in the National Collection of Industrial Bacteria (accession number NCIB 11596).

Clostridium puniceum sp.nov.

pu.ni'ce.um. L.adj. *puniceus* pink.

Straight rods 0.6×1.8 to $4.2 \mu\text{m}$ occurring singly, in pairs or in short chains. Motile with peritrichous flagella. Gram-negative. Forms boat-shaped, clostridial cells containing granulose and in which spores are subsequently produced in a subterminal position. Free spores are oval and surrounded by an extensive exosporium in which are arrays of subunits showing a hexagonal periodicity.

On potato infusion agar after incubation under H_2/CO_2 (9:1, v/v) for 4 d at 25°C typical colonies are circular, 1 to 2 mm in diameter, convex, amorphous, smooth, undifferentiated, edge entire, pale pink, opaque, viscid and difficult to emulsify.

The optimum temperature for growth is 23 to 33°C and the range is 7 to 39°C .

No growth occurs in media without a fermentable carbohydrate. Raw potato tissue is macerated and pectate, starch, gelatin and casein are hydrolysed; some strains fail to hydrolyse casein. Major fermentation products from glucose are butyric and acetic acids and butanol.

The following carbohydrates are used as carbon sources for growth and acid production: amygdalin, arabinose, cellobiose, fructose, galactose, glucose, lactose, maltose, melibiose, raffinose, salicin, starch, sucrose, trehalose and xylose. Some strains are also able to utilize glycogen, inulin, mannitol, melezitose and ribose, but fail to utilize raffinose. Dulcitol, glycerol, inositol, rhamnose, sorbitol and sorbose are not utilized.

Type strain: NCIB 11596 (= BL70/20)

The G + C content of the DNA of the type strain is 28 to 29 mol % (by T_m).

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