Characterization of a Propionic Acid Producing Actinomycete, *Actinomyces propionicus*, Sp.nov.

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SUMMARY

An anaerobic actinomycete isolated from the lachrymal duct of a case of human lachrymal canaliculitis is described. Although the organism was described previously as *Actinomyces israelii*, more recent observations show it differs significantly from members of this genus in metabolism, physiology and cell-wall composition. The organism is similar to the *Actinomyces* in its production of true branching mycelial elements *in vivo* and *in vitro*, catalase negativity, pathogenicity for experimental animals, and amino acid cell-wall composition. It is similar to the propionic acid bacteria in its fermentation of glucose to propionate, acetate, and CO₂, its morphological variation, formation of a dull orange colour, and the presence of diaminopimelic acid in its cell wall. Other characteristics such as the formation of DL-lactic acid, absence of a CO₂ requirement either anaerobically or aerobically, inability to ferment glycerol or lactate, and sugar and amino sugar composition of the cell wall also serve to differentiate this organism from other strains of *Actinomyces* and from *Propionibacterium*. In view of these observations the organism is reclassified and named *A. propionicus*, sp.nov. The possible phylogenetic relation of *A. propionicus* to certain other branching filamentous organisms is discussed.

INTRODUCTION

From studies of human canaliculitis, Pine & Hardin (1959) and Pine, Hardin, Roberts & Turner (1960) have described two of the etiological agents as *Actinomyces israelii*. Both organisms were found to possess morphological and physiological characters typical of the species. However, one of the organisms, strain 699, fermented glucose to form propionic acid in addition to the acetic, formic, lactic, and succinic acids described for other strains (Pine & Howell, 1956). Because of this difference, the morphological, physiological and biochemical characteristics of strain 699 have been studied further. Although strain 699 possessed many of the characters of recognized species of *Actinomyces* (Bergey's Manual, 1957; Howell, Murphy, Paul & Stephan, 1959; Pine, Howell & Watson, 1960; Waksman, 1961) the more recent findings showed it had major differences in metabolism, physiology, and cell-wall composition. In many respects it was closely related to members of the genus *Propionibacterium*. After consideration of these findings we were unable to assign the organism to a previously described species of either of these genera. As a result, we have concluded that the organism warrants inclusion in the genus *Actinomyces* and propose the name *Actinomyces propionicus*, sp.nov.

METHODS

Actinomyces propionicus (*A. israelii*, strain 699) was isolated as described previously by Pine & Hardin (1959). Sources of other species of *Actinomyces* were described by Pine, Howell & Watson (1960). *Propionibacterium arabinosum*, 4965, and *P. pentosaceum*, 4875, were obtained from the American Type Culture Collection. The Casitone medium of Pine & Watson (1959) with no added lipoic acid was used throughout this study. The procedures for the preparation of inocula and growth were those described by Howell & Pine (1956).

Methods of growth studies

Amounts of growth were determined by preparing a 1/10 or 1/20 dilution of homogenized cultures with distilled water and reading the absorbancy at 660 m μ on a Spectronic-20 colorimeter against a corresponding dilution of uninoculated medium. One growth unit = optical density unit divided by the dilution, and was equivalent to 0.3 mg. dry wt. organism. Forty-eight hr. cultures of *Actinomyces bovis* (P1S) and *A. naeshundii* (279) and 72 hr. cultures of *A. propionicus* and *A. israelii* (895) were diluted with sterile distilled water to an optical density = 0.5 on a Spectronic-20 colorimeter. Test tubes containing 5 ml. Casitone medium were inoculated with one drop of this suspension. Anaerobic cultures were incubated under a pyrogallol + Na₂CO₃ seal. Aerobic cultures were either unsealed or sealed with KH₂PO₄(M) + Na₂CO₃ (10 % w/v) as indicated and incubated on a rotary shaker. All incubations were conducted at 37°.

The methods used to determine fermentative and physiological characters were as previously described (Pine & Howell, 1956). The size of inoculum required for aerobic growth was determined according to the procedure of Howell *et al.* (1959).

For pathogenicity studies, organisms from 72 hr. cultures of *Actinomyces* spp. and from 48 hr. cultures of *Propionibacterium* were harvested by centrifugation and washed twice with sterile distilled water. Packed organisms (0.5 ml.) were brought to a volume of 5 ml. with physiological saline (NaCl, 0.85 %, w/v). White female mice ranging from 5 to 6 weeks of age were inoculated intraperitoneally with 0.2 ml. or 0.5 ml. of this suspension, depending upon the experiment. After 18 days animals were sacrificed and examined for internal lesions. When lesions were found these were recorded, isolation plates were streaked with pus, and Gram stains of the pus made. Isolations were made on brain heart infusion agar (Difco) plates which were incubated 1-3 days under an atmosphere of 5 % (v/v) CO₂-95 % (v/v) N₂.

For product analysis fermentations were carried out in duplicate; one of each duplicate contained uniformly labelled ¹⁴C-glucose of known specific activity. All fermentation vessels were inoculated with 2 mg. equivalent of dry wt. organism and incubated for 3 days at 37°. For anaerobic studies, the organisms were grown in fermentation tubes (Pine & Howell, 1956) containing 50 ml. of medium. Tubes were sealed with 1 ml. each of Na₂CO₃ + pyrogallol or NaOH + pyrogallol. For aerobic studies organisms were grown on a rotary shaker in a 160 ml. Erlenmeyer flask containing 50 ml. medium and stoppered with a vaccine stopper. At the end of the

incubation period 1 ml. of 50 % (w/v) CO₂-free NaOH was injected through the vaccine stopper to make the liquor strongly alkaline.

Carbon dioxide was determined manometrically by the method of Peters & Van Slyke (1932). Volatile acids were quantitatively estimated by the method of Friedemann (1938) as modified by Rabinowitz & Barker (1956). After formate oxidation, propionate and acetate concentrations were calculated by solving simultaneous equations based on Duclaux values. Glucose was determined by the method of Folin & Malmros as given by Umbreit, Burris & Stauffer (1957).

Succinate and lactate were quantitatively estimated from fermentations conducted with radioactive glucose. They were collected by 48 hr. ether extraction and separated and identified by means of a Dowex-1 column with a formic acid gradient according to a method modified from that of Busch, Hurlbert & Potter (1952). This method effects repeatable separation of lactic and succinic acids with no overlapping of peaks. The formic acid eluant was removed by evaporation on a steam bath, and the non-volatile acids were quantitatively determined by titration with 0.1 N-NaOH to the phenol red end-point.

Glucose incorporation into cellular material was calculated from organisms grown on ¹⁴C-labelled glucose which were collected by centrifugation, washed 5 times with distilled water, pipetted on to planchets and dried under infrared heat. Samples were counted at infinite thinness. Radioactivity was measured with a continuous flow counter equipped with a 'micromil' window (Nuclear-Chicago).

The lactic acid used for determination of optical rotation was obtained from a 6 l. flask of fermentation liquor. The flask contained a modified Casitone medium in which acid-hydrolysed casein (Nutritional Biochemical Co., Cleveland, Ohio) was substituted for Casitone and the minor-element solution omitted. Lactate was collected by 48 hr. ether extraction and crystallized as the zinc salt (Pederson, Peterson & Fred, 1926). Optical activity was determined with a solution of zinc lactate in a Model BO polarimeter (O.C. Rudolph and Sons Inc., Caldwell, N.J.).

Cell walls were isolated and acid-hydrolysed according to the method of Cummins & Harris (1956). In most cases amino acids and amino sugars were identified solely by R_f values on two-dimensional paper chromatograms developed as described by these investigators. Diaminopimelic acid and lysine were more definitely identified by co-chromatography with known compounds. Sugars as well as amino sugars were identified by R_f values from unidirectional paper chromatograms developed in butanol + pyridine + water (6 + 4 + 3 by vol) and butanol + acetic acid + water (5 + 1 + 2 by vol). Sugar and amino sugar spots were located on duplicate sheets and were characterized by their comparative reactions using a modified silver nitrate dip (Dr R. W. Wheat, unpublished results) and a ninhydrin spray (0.25 %, w/v, ninhydrin in 95 %, v/v, ethanol in water).

A culture of *Actinomyces propionicus* 699 has been deposited in the American Type Culture Collection, 2112 M Street, N.W., Washington, D.C., under the number ATCC 14157.

RESULTS

As a result of preliminary fermentation studies, it became evident that *Actinomyces propionicus* fermented glucose in a manner typical of many species of *Propionibacterium*. Moreover, certain morphological and physiological characteristics

common to the propionibacteria became apparent. Although a brief description of the morphological and physiological characters of *A. propionicus* was presented previously (Pine & Hardin, 1959), it subsequently became necessary to extend these studies and to compare the organism directly to known species of *Actinomyces* and *Propionibacterium*.

Cellular morphology

As shown in Pl. 1, fig. 1, *Actinomyces propionicus* formed branched mycelia typical of other members of the genus (Howell *et al.* 1959; Pine, Howell & Watson, 1960). However, when the organism was grown in 1 l. volumetric flasks under Na_2CO_3 +pyrogallol seals to prepare organisms for enzymic studies, a marked change in morphology occurred. For reasons which are not known, *A. propionicus* changed under these conditions from a short branching rod in the initial stages of growth to a long thread-like element which tangled to form a cotton-like mass (Pl. 1, fig. 2). As growth progressed occasional bulbous ends were observed on some cells. In sugar fermentation tests the sugar fermented greatly influenced the morphology. After 10–14 days of incubation in glucose fermentation tubes, forms identical with those of *Propionibacterium pentosaceum* were observed (Pl. 1, fig. 3). Here the individual cells in a hyphal element swelled with apparent spheroplast formation. With raffinose as a substrate virtually all cells rounded and swelled to form solid masses of spherical cells (Pl. 1, fig. 4). Although the number of spherical cells or hyphal-like threads which were formed varied from one substrate to another, it apparently was not directly related to the amount of growth or acid produced on any given substrate. While further studies were necessary to determine what factors influenced the morphology of the organism, the morphological similarity of *A. propionicus* to certain of the propionic acid bacteria as originally described by Van Niel (1938) and by Hitchner (1932, 1934) was quite obvious. However, it may be pointed out that spherules such as those shown in Pl. 1, fig. 4, were much larger than any observed with propionibacteria. In addition, they were rigid structures resistant to the osmotic effects of distilled water or 20 % (w/v) sucrose.

Colony morphology

Of the taxonomic criteria used in the identification of species of *Actinomyces*, the morphology of the micro-colony is of paramount importance (Wright, 1905; Emmons, 1935; Howell *et al.* 1959; Pine, Howell & Watson, 1960). Consequently, the colony morphology of *Actinomyces propionicus* was compared to that of *Propionibacterium pentosaceum* and *P. arabinosum* (Pl. 2, figs. 6, 7, 8, 9). Under low power the mycelial nature of *A. propionicus* was readily apparent, while the colony of *P. arabinosum* was round with an unbroken periphery. Under high power, the colony of *A. propionicus* was definitely mycelial with long hyphal elements which branched repeatedly (Pl. 2, fig. 8). Under the same conditions colonies of *P. arabinosum* and *P. pentosaceum* appeared to be mycelial although the hyphal elements were short and branching was not apparent (Pl. 2, fig. 9). Moreover, when a coverslip was placed over these colonies, they immediately disintegrated to form a free-flowing mass of individual cells having no semblance of colonial structure. On the other hand, *A. propionicus* tenaciously maintained its intact mycelial structure

under these conditions. These characteristics indicated that while *A. propionicus* produced definite mycelial colonies, cells of the propionic acid bacteria were very loosely connected and did not form a true mycelium.

Relative pathogenicity

To the authors' knowledge there have been no experimental pathogenicity studies conducted with species of *Propionibacterium*. Consequently, comparative pathogenicity studies were made with species of *Propionibacterium* and *Actinomyces*. The results of animal inoculations with *Actinomyces propionicus* strongly indicated that it was no less pathogenic than any of the other species of *Actinomyces* tested (Table 1). The results given in Table 1 suggested, and this was supported by several additional experiments, that *A. propionicus* was slightly more pathogenic than the other strains of *Actinomyces* tested. On the other hand, failure to observe typical mycelial elements or any Gram-positive organisms in the lesions of animals inoculated with species of *Propionibacterium*, and the failure to isolate these organisms, showed that the propionibacteria were non-pathogenic. The fact that spontaneous infections in animals or man due to propionibacteria have not been reported (Topley & Wilson, 1957) support these observations. From the appearance of *A. propionicus* in animal lesions (Pl. 1, fig. 5) there was no doubt that it was similar morphologically to other species of *Actinomyces* in clinical material although it has often shown a greater propensity for being thread-like (Pine & Hardin, 1959).

Sugar fermentations

The results of the utilization of various substrates for growth and acid production by *Actinomyces propionicus* and related organisms are presented in Table 2. The organism was found to follow the same general pattern of other *Actinomyces* in these respects. While there were other major differences between the anaerobic actinomycetes and members of the genus *Propionibacterium*, the complete inability of *Actinomyces* species to ferment and utilize glycerol for growth and acid production was the most pronounced (Howell *et al.* 1959; Pine, Howell & Watson, 1960). All species of *Propionibacterium* ferment glycerol and lactate with the formation of acid (Bergey's Manual, 1957); *A. propionicus* could not use these substrates for growth in many repeated experiments. In its fermentation of sugars, *A. propionicus* was similar to *A. naeslundii* for it fermented raffinose but failed to ferment xylose (Howell *et al.* 1959). The ability to ferment mannitol was not shown by strains of *A. naeslundii* whereas mannitol always supported good growth of *A. propionicus* although acid production was variable.

Physiological characteristics

The compared physiological characteristics of the *Actinomyces* and *Propionibacterium* spp. are recorded in Table 3. Colonies of *A. propionicus* had a dull orange colour when grown on the surface of aerobic or anaerobic tubes, whereas all other species of *Actinomyces* remained white. *A. propionicus* also produced large amounts of H_2S ; it was similar to the facultative anaerobe, *A. naeslundii*, in that both reduced nitrate to nitrite. *A. propionicus* was consistently catalase negative. On the other hand, the propionic acid bacteria were catalase positive as previously described (Bergey's Manual, 1957; Van Niel, 1928).

Table 1. *Comparative pathogenicity of species of Actinomyces and Propionibacterium*

Organism	Size of inoculum packed cells ml./mouse	Mice incubated (days)	Mice inoculated (r.o.)	Mice having abscesses (no.)	Description of infections*	Gram + filaments no. of mice	Positive isolation no. of mice†
<i>Actinomyces propionicus</i> 699	0.05	18	5	5	All mice showed multiple lesions on stomach, kidney, intestinal tract, and/or liver. One mouse had 2 cm. encapsulated abscess on lower peritoneal wall with numerous lesions on dorsal part of liver	5	n.d.
	0.05	33	3	3	One mouse with massive intestinal abscess adherent to spleen, multiple lesions throughout. Second mouse with two large abscesses, third with single small abscess on lower intestine	3	n.d.
<i>A. naestlundii</i> 279	0.05	18	6	3	Two mice each with encapsulated abscess diameter greater than 2 cm., one on intestine, one on liver. Multiple small abscesses in addition. Third mouse with large intestinal abscess and small lesions on liver and mesentery	3	n.d.
<i>A. bovis</i> P18	0.05	32	6	1	Small lesions adherent to intestinal wall	1	n.d.
<i>A. propionicus</i> 699	0.02	25	4	4	Small abscesses at point of inoculation and on liver	4	4
	0.02	32	4	4	As above but two mice each with single encapsulated lesion 0.5–0.7 cm. in diameter. One in liver, one on the peritoneal wall	4	4
<i>A. naestlundii</i> 279	0.02	25	5	2	One with two small lesions on peritoneal wall, one with single lesion at point of inoculation	2	n.d.
	0.02	32	5	5	Small lesions on peritoneal cavity or at point of inoculation	5	5
<i>A. israelii</i> 895	0.02	25	4	3	One mouse with large encapsulated abscess attached to liver and stomach. Second with multiple lesions throughout abdominal cavity. Third with three small lesions on liver and one on kidney	4	n.d.
	0.02	32	3	0	No infections	0	n.d.
<i>Propionibacterium arabinosum</i> ATCC 4965	0.02	21	7	4	One mouse with multiple lesions on liver, three mice with single small lesion at point of inoculation	0	0
<i>Propionibacterium pentosaceum</i> ATCC 4875	0.02	21	6	3	One mouse with multiple lesions on liver, two mice with single lesion at inoculation site	0	0

* Pus from the largest and most developed lesions was taken from infected mice and mounted in 10% (w/v) KOH for microscopic examination. Although in some cases mycelial clumps were observed, typical sulphur granules were not seen in any of the preparations.

† n.d. = Quantity not determined.

Table 2. *Comparison of the growth and fermentative ability of Actinomyces propionicus with other Actinomyces and Propionibacterium spp.*

+ = Acid and no gas (bromocresol purple indicator). Relative growth expressed in units ranging from a minimum = 1 to a maximum = 5. Duplicate tubes containing 5 ml. were inoculated with 1 drop of an homogenized 48–72 hr. culture and incubated under pyrogallol + Na₂CO₃ seal for 7–10 days at 37°. Basal medium was the Casitone medium with 1.0% (w/v) substrate. Strain numbers are indicated below the name of each species.

Substrate	Organism											
	<i>A.</i>		<i>A.</i>		<i>A.</i>		<i>A.</i>		<i>P.</i>		<i>P.</i>	
	<i>propionicus</i>		<i>israelii</i>		<i>bovis</i>		<i>naeslundii</i>		<i>arabinosum</i>		<i>pentosaceum</i>	
	(699)		(895)		(P1S)		(279)		(ATCC 4965)		(ATCC 4875)	
	A.*	G.†	A.	G.	A.	G.	A.	G.	A.	G.	A.	G.
Glucose	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5
Fructose	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5
Mannose	+, +	5, 5	n.d.‡	n.d.	n.d.	n.d.	n.d.	n.d.	+, +	5, 5	+, +	4, 4
Galactose	+, +	3, 3	±, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	4, 4	+, +	5, 5
Lactose	+, +	5, 5	+, +	3, 3	+, +	5, 5	+, +	5, 5	+, +	4, 4	+, +	5, 5
Maltose	+, ±	5, 5	±, +	5, 5	+, +	5, 5	±, +	5, 5	+, +	4, 4	+, +	4, 4
Mannitol	+, –	5, 5	±, ±	4, 4	–, –	1, 1	–, –	5, 5	+, +	4, 4	+, +	4, 4
Sucrose	±, ±	5, 5	+, +	5, 5	+, +	5, 5	+, +	5, 5	+, +	4, 4	+, +	4, 4
Xylose	–, –	1, 1	+, +	5, 5	–, –	1, 1	–, –	3, 3	–, –	2, 2	+, +	4, 4
Raffinose	±, ±	5, 5	±, +	5, 5	–, –	1, 1	+, +	5, 5	–, –	1, 1	+, +	4, 4
Arabinose	+, +	5, 5	–, –	1, 1	–, –	1, 1	–, –	1, 1	–, –	1, 1	+, +	4, 4
Ribose	±, ±	3, 3	+, +	5, 5	–, –	1, 1	–, –	1, 1	+, +	4, 4	+, +	4, 4
Rhamnose	–, –	3, 3	–, –	3, 3	±, ±	5, 5	–, –	1, 1	±, ±	3, 3	+, +	3, 3
Salicin	–, –	2, 2	±, +	5, 5	±, ±	1, 1	±, ±	5, 5	±, ±	3, 3	±, ±	3, 3
Starch	±, ±	5, 5	±, ±	5, 5	+, +	5, 5	±, ±	5, 5	–, –	2, 2	±, ±	4, 4
Glycogen	–, –	1, 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	–, –	1, 1	–, –	2, 2
Lactic acid	–, –	1, 1	–, –	1, 1	–, –	1, 1	–, –	1, 1	+, +	5, 5	+, +	5, 5
Glycerol	–, –	1, 1	–, –	1, 1	–, –	1, 1	–, –	1, 1	+, +	5, 5	+, +	5, 5
Cellulose	–, –	1, 1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* A = Acid production. † G. = Growth. ‡ n.d. = quantity not determined.

Carbon dioxide requirement for anaerobic and aerobic growth

All species of *Actinomyces* have been shown to require CO₂ for maximum anaerobic growth, while *A. naeslundii*, a facultative anaerobe, manifests a similar requirement aerobically (Pine & Howell, 1956; Howell *et al.* 1959). Consequently, the CO₂ requirement for *A. propionicus* was investigated, both anaerobically and aerobically. As seen in Table 4, this organism was similar to *A. naeslundii* in that it grew well aerobically although anaerobic growth was induced by a smaller inoculum than that required for aerobic growth. In contrast to *A. naeslundii* (Howell *et al.* 1959) CO₂ had no stimulating effect on aerobic growth of *A. propionicus*. A comparison of the growth curves of *A. naeslundii* grown aerobically in the presence and absence of added CO₂ with those of *A. propionicus* showed conclusively that *A. propionicus* did not have the CO₂ requirement for aerobic growth so consistently demonstrated for *A. naeslundii* (Fig. 1). Similarly, fermentations carried out under a NaOH + pyrogallol seal revealed that *A. propionicus* did not require CO₂ for anaerobic growth (Table 5).

Table 3. *Physiological characteristics of Actinomyces propionicus and Actinomyces and Propionibacterium spp.*

Physiological tests were carried out as described previously (Pine & Howell, 1956). With the exception of litmus milk, basal medium was the Casitone medium with 1.0% (w/v) glucose. Conditions of growth as given in legend to Table 2.

Character	<i>A. propionicus</i> (699)	<i>A. israelii</i> (895)	<i>A. bovis</i> (P1S)	<i>A. naeshlundii</i> (279)	<i>P. arabinosum</i> (4965)	<i>P. pentosaceum</i> (4875)
Catalase	—*	—	—	—	+	+
Colour	Dull orange	White	White	White	Orange-yellow	Cream
Gelatin liquefaction	—	—	—	—	—	—
Starch hydrolysis	—	—	+	—	—	—
Indole production	+	+	+	+	—	—
Nitrate reduced to nitrite	+	—	—	+	+	—
Acetoin production	—	—	—	—	—	—
H ₂ S formation	+	—	—	—	±	—

* Catalase was negative whether the organism was grown aerobically or anaerobically in the presence or absence of added haemin.

Table 4. *Effect of inoculum size on anaerobic and aerobic growth of Actinomyces propionicus*

A 48 hr. culture of *A. propionicus* was homogenized and diluted with sterile distilled water to an optical density = 0.5. This suspension was serially diluted with water as indicated. Duplicate tubes containing 5 ml. of Casitone medium with 0.5% (w/v) glucose were inoculated with 1 drop of each dilution and incubated for 84 hr. at 37°. Aerobic cultures were grown on a rotary shaker. Relative growth expressed for duplicate tubes in units ranging from a minimum = 1 to a maximum = 5; — signifies no growth.

Growth conditions	Dilution used for inoculum									
	10 ⁻¹	10 ⁻²	10 ⁻³	10 ⁻⁴	10 ⁻⁵	10 ⁻⁶	10 ⁻⁷	10 ⁻⁸	10 ⁻⁹	
	Relative growth									
Anaerobic:										
Na ₂ CO ₃ + pyrogallol seal	5, 5	5, 5	4, 4	4, 4	3, 3	2, 1	2, 1	2, 1	2, 1	
Aerobic:										
Na ₂ CO ₃ + KH ₂ PO ₄ seal	5, 5	5, 5	3, 2	5, 2	1, —	—, —	—, —	—, —	—, —	—, —
Aerobic: no seal	5, 5	5, 5	2, 2	1, 1	—, —	—, —	—, —	—, —	—, —	—, —

Comparative growth of species of Actinomyces

Figure 2 shows the comparative growth curves of species of *Actinomyces*. Anaerobically the rate of growth of *A. propionicus* was not usually as great as that of *A. naeshlundii* and was usually less than that of *A. bovis* or a granular strain of *A. israelii*. However, as shown in Fig. 2 the yields of *A. propionicus* grown anaerobically were comparable to those of *A. naeshlundii* and much greater than those of *A. israelii* and *A. bovis*. Generally, species of *Actinomyces* utilize only 25–30 μ mole glucose/ml. medium (Pine & Howell, 1956). This has been shown to be due to sensitivity of these organisms to the acid formed; greater glucose utilization with

increased yields of growth occurred in an adequately buffered medium (Buchanan & Pine, unpublished results). However, *A. propionicus* utilized only 16 μ mole glucose/ml. in Casitone medium, yet the growth yields as judged by optical density measurements were greater than those observed for the other species (Fig. 2). This ability of *A. propionicus* to form more organisms from less glucose would appear to be related to a more efficient pathway of glucose dissimilation (Bauchop & Elsdén, 1960). Usually growth yields of *A. naeslundii* doubled in the presence of oxygen (Pine & Howell, 1956; Buchanan & Pine, unpublished results). However, without changing the medium, growth of *A. propionicus* in air did not increase significantly (Figs. 1, 2), although all the glucose (27 μ mole/ml.) was utilized (Table 5). The reason for this is unknown at present; it may be due to the limitation of growth factors in the medium.

Fig. 1

Fig. 1. Comparative aerobic growth of *Actinomyces propionicus* and *A. naeslundii* with and without added CO₂.

Fig. 2

Fig. 2. Comparative anaerobic growth of species of *Actinomyces*.

Fermentation balances

The results of the glucose fermentations of *Actinomyces propionicus* and related organisms are given in Table 5. Anaerobically, in the presence or absence of added CO₂, *A. propionicus* produced principally CO₂, acetic and propionic acids, with a ratio of products that was typical of many strains of *Propionibacterium*. However, the reported utilization of glucose by *Propionibacterium* spp. was much greater than that observed for *A. propionicus* (Wood & Werkman, 1936). From 70 to 80 % of the total carbon utilized could be accounted for by these products. While their concentrations were quite small, lactate and succinate were definitely produced under these conditions, although the amounts of lactate produced varied. After consideration of the data of Wood & Werkman (1936) and Wood, Stone & Werkman

(1937) it was concluded that there was no significant qualitative or quantitative difference between glucose fermentations of *A. propionicus* and species of *Propionibacterium* other than the total amount of glucose fermented.

Aerobically in the absence of added CO₂, *Actinomyces propionicus* fermented glucose to yield stoichiometric amounts of acetic acid and CO₂. While there was a small percentage of carbon which remained unaccounted for, this was not present in the ether-extractable non-volatile fraction. Absence of radioactivity in any remaining fraction other than whole cells indicated that no other products were formed in significant amounts.

Table 5. *Glucose fermentation balances for Actinomyces propionicus and related organisms*

Organism	Growth conditions	Glucose fermented (μ mole/ ml.)	Expressed in μ mole/100 μ mole glucose fermented						
			CO ₂ fixed	CO ₂ produced	Acetate	Propio- nate	Formate	Lactate	Succinate
<i>A. propionicus</i>	Anaerobic: Na ₂ CO ₃ + pyrogallol seal	15.9	—	53.5	55.3	114.5	4.4	2.5	3.1
	—	16.4	—	54.9	53.7	111.0	0.0	2.4*	3.0
	Anaerobic: NaOH+ pyrogallol seal	14.4	—	n.d.	71.5	118.7	0.0	2.8	4.9
	—	13.9	—	n.d.	48.9	133.8	9.4	2.9*	5.0
	Aerobic: no seal	27.0	—	175.9	144.8	0.0	0.0	0.5	0.5
—	—	27.5	—	169.4	157.4	0.0	0.0	0.5*	0.5
<i>A. israelii</i> , 295†	Anaerobic: Na ₂ CO ₃ + pyrogallol seal	26.6	32.7	—	23.7	0.0	29.3	139.5	32.3
<i>Propionibacterium arabinosum</i> (34 W ₁)§	Anaerobic: under N ₂ -atmosphere	145.3	—	63.6	17.8	139.1	—	—	13.2
<i>P. pentosaceum</i> (49 W ₃)§	Anaerobic: under N ₂ -atmosphere	155.9	—	52.3	16.6	127.8	—	—	21.1
<i>P. shermanii</i> (52 W ₃)§	Anaerobic: under N ₂ -atmosphere	163.9	—	47.0	45.9	106.1	—	1.5	15.0
Expressed in μ mole/100 μ mole glucose fermented									
			Non- reducing material		Cellular glucose incorpora- tion	Ratio: CO ₂ / acetate	Ratio: propio- nate/ acetate	Carbon recovery (%)	Redox index
<i>A. propionicus</i>	Anaerobic: Na ₂ CO ₃ + pyrogallol seal	15.9	—	—	15.7	0.97	2.07	104.4	1.00
	—	16.4	—	—	15.2*	1.02	2.07	101.0	1.02
	Anaerobic: NaOH+ pyrogallol seal	14.4	—	—	14.6	—	1.66	102.4†	—
	—	13.9	—	—	15.1*	—	2.74	104.7†	—
	Aerobic: no seal	27.0	—	—	15.6	1.21	—	93.2	—
	—	27.5	—	—	15.3*	1.08	—	96.0	—
<i>A. israelii</i> , 295†	Anaerobic: Na ₂ CO ₃ + pyrogallol seal	26.6	—	—	—	—	—	98.7	1.06
<i>Propionibacterium arabinosum</i> (34 W ₁)§	Anaerobic: under N ₂ -atmosphere	145.3	2.8	—	3.59	7.82	97.6	1.01	—
<i>P. pentosaceum</i> (49 W ₃)§	Anaerobic: under N ₂ -atmosphere	155.9	2.8	—	3.15	7.70	95.0	0.98	—
<i>P. shermanii</i> (52 W ₃)§	Anaerobic: under N ₂ -atmosphere	163.9	4.1	—	1.02	2.33	91.2	1.02	—

* Assumed values from preceding radioactive glucose fermentation. † Co₂ not considered in fermentation balance. ‡ Data of Pine & Howell (1956). § Data of Wood & Werkman (1936). n.d. = quantity not determined.

Optical rotation of lactic acid

Although the amount of lactic acid produced by *Actinomyces propionicus* varied, it was generally quite small in large fermentations. From 6 l. of fermentation liquor about 6 g. zinc lactate were obtained, of specific rotation +0.10. Because of relations to be discussed subsequently, the lactic acid formed by *Bacterionema*

Table 6. *Cell-wall composition of Actinomycetes propionicus and related organisms*

	Arabinose	Rhamnose	Galactose	Glucose	Mannose	Fucose	Ribose	Glucosamine	Galactosamine	Muramic acid	Aspartic acid	Alanine	Glutamic acid	Glycine	Lysine	Diaminopimelic
<i>Actinomyces propionicus</i>	—	—	++	++	—	—	tr.	+	+	?tr.	+	+	+	—	tr.	+
<i>A. israelii</i> *	—	—	+	—	—	—	NR	+	—	+	·	+	+	·	+	·
<i>A. bovis</i> *	—	—	tr.	—	tr.	+	NR	+	—	+	+	+	+	·	+	·
NCTC 9430	—	+	+	—	—	+	NR	+	—	+	+	+	+	·	+	·
NCTC 9431	—	+	+	—	—	—	NR	+	—	+	+	+	+	·	+	·
<i>Propionibacterium pentosaceum</i> *	—	—	+	+	tr.	—	NR	+	+	+	·	+	+	+	·	+
NCIB 8070	—	—	+	++	tr.	—	NR	+	tr.	+	·	+	+	+	·	+
<i>P. jensenii</i> *	—	+	+	++	tr.	—	NR	+	tr.	+	·	+	+	+	·	+
NCIB 8069	—	—	—	+	—	—	NR	tr.	—	NR	—	+	+	—	—	+
<i>Bacterionema matruchotii</i> †‡	+	—	—	+	—	—	NR	tr.	—	—	—	+	+	—	—	+
<i>(Leptotrichia dentium)</i>	—	—	+	—	—	NR	NR	·	tr.	·	tr.	++	+	tr.	+	—
<i>Nocardia salivae</i> §	—	—	+	—	—	NR	NR	·	tr.	·	tr.	++	+	tr.	+	—

tr. = Trace; NR = not reported; · = amino acids not considered as major constituents.

* Data of Cummins & Harris (1956, 1958) for representative strains. † Data of Davis & Baird-Parker (1959). ‡ Synonymy of names given by Gilmour, Howell & Bibby (1961). § Data of Davis & Freer (1960).

matruchotii (*Leptotrichia dentium*); Baird-Parker & Davis, 1958 (Gilmour, Howell & Bibby, 1961) was converted to the zinc salt and optically assayed as described for *A. propionicus*. A specific rotation of -0.63 was found. Since the zinc salt of the pure optically active isomer has a specific rotation of $+$ or -8.25 (Pederson, Peterson & Fred, 1926) and because of the low solubility of the zinc lactates of the two organisms tested, it was concluded that both *B. matruchotii* and *A. propionicus* formed DL-lactic acid.

Cell-wall analysis

Cummins & Harris (1958, 1959), Davis & Baird-Parker (1959) and Davis & Freer (1960) have shown cell-wall composition to be a general taxonomical character relevant to the classification of Actinomycetales. Consequently, cell walls of *Actinomyces propionicus* were isolated and analysed. The results of this analysis and those of several related organisms as reported by other workers are given in Table 6. Alanine, glutamic, aspartic, and diaminopimelic acids were the major amino acid components of the *A. propionicus* cell-wall hydrolysate, while there was a small but definite amount of lysine. There appeared to be a trace of muramic acid as suggested by the colour formed with ninhydrin and its position on the paper, but this was not further identified. Galactose and glucose and glucosamine and galactosamine represented the major sugar and amino sugar constituents, respectively. No attempt was made to determine the stereoisomeric form of the diaminopimelic acid.

DISCUSSION

From the results of experiments comparing pathogenicity of *Propionibacterium* spp. and *Actinomyces* spp., it is obvious that *Actinomyces propionicus* is readily classified in the genus *Actinomyces*, although pathogenicity is not usually a taxonomic characteristic used to separate genera. Stanier & Van Niel (1941) and Howell *et al.* (1959) emphasized morphology as the primary diagnostic character pertaining to the classification of members of the Actinomycetales. Primarily on the basis of its colony morphology and its morphological appearance in animals, *A. propionicus* was initially classified as a strain of *A. israelii* (Pine & Hardin, 1959). In the original spontaneous infection and in inoculated animals the long branching mycelial threads were typical of strains of *A. bovis*, *A. israelii* and *A. naeslundii*.

On the basis of cellular morphology *in vitro*, there is little to distinguish *Actinomyces propionicus* from *Propionibacterium arabinosum* or *P. pentosaceum*, although during the logarithmic growth phase there is a much greater tendency for *A. propionicus* to form true branching rods and hyphal elements. Subsequent to the stationary phase of growth or dependent upon cultural conditions and media, the propionibacteria form spherical cells which may be normal or greatly enlarged in diameter. In this respect the cells of *A. propionicus* may be identical with those of *P. arabinosum* (Skerman, 1959), although the cells of *A. propionicus* continue to enlarge under certain conditions of substrate and age to form huge spheres not unlike the cystites of *Arthrobacter* species (Chaplin, 1957; Skerman, 1959). Although there have been no direct experiments to determine the nature of these morphological changes, the impression gained from general observation strongly suggests that these coccoidal or swollen forms are a result of abnormal cell-wall formation and do not

constitute a normal phase of the organism's growth. Similar types of cells have been observed in cultures of *A. bovis* (Pine, Howell & Watson, 1960) although these lacked the rigidity of structure observed in cells of *A. propionicus*.

Perhaps one of the strongest diagnostic characteristics identifying the anaerobic actinomycetes infecting man is the formation within a few days of small delicate mycelial colonies on the agar surface. In this regard, *Actinomyces propionicus* formed a colony typical of that formed by strains of *A. israelii*, whereas such a colonial structure was completely absent in the colonies of *Propionibacterium arabinosum* and *P. pentosaceum*. However, similar to the colonies of *P. arabinosum* (Bergey's *Manual*, 1957), the colonies of *A. propionicus* grown aerobically or anaerobically on the surface of agar media gradually assumed a dull orange colour within several weeks while all other strains of *Actinomyces* spp. remained white or cream.

In its physiological and fermentative aspects *Actinomyces propionicus* does not show distinctive characters of its own but combines several of the identifying characteristics of the genera *Actinomyces* and *Propionibacterium*. Like all members of the genus *Actinomyces* (Pine & Howell, 1956; Howell *et al.* 1959; Pine, Howell & Watson, 1960) *A. propionicus* does not ferment lactic acid or glycerol, whereas these substrates are fermented by all *Propionibacterium* spp. (Bergey's *Manual*, 1957). Like most strains of *A. naeslundii* (Howell *et al.* 1959), *A. propionicus* ferments raffinose but does not ferment xylose or mannitol, although growth of *A. propionicus* on mannitol is invariably good and substantial acid formation is occasionally observed. Like all strains of *A. naeslundii* tested, *A. propionicus* reduces nitrates to nitrites.

Species of *Actinomyces* are generally recognized as being catalase negative (Suter, 1956; Hazen & Little, 1958; Pine & Howell, 1956; King & Meyer, 1957; Howell *et al.* 1959; Pine, Howell & Watson, 1960). Similarly *Actinomyces propionicus* was found to be catalase negative. Since catalase may be an inducible enzyme (Clayton, 1960) or be dependent upon the presence of essential factors (Biberstein & Gills, 1961) many attempts were made to demonstrate the formation of catalase by *A. propionicus* grown anaerobically or aerobically in the presence and absence of added haemin; these attempts failed and catalase formation was not demonstrated. On the other hand, catalase activity of *Propionibacterium arabinosum* and *P. pentosaceum* has been described as being extremely limited (Bergey's *manual*, 1957). Although this was true of *P. arabinosum* when it was received, the initial catalase activity of *P. pentosaceum* was high. However, with further culturing on the Casitone medium, the catalase activity of *P. arabinosum* also became strongly positive.

All strains of *Actinomyces* studied previously required substrate amounts of CO₂ to ferment glucose, forming formic, acetic, lactic and succinic acids (Pine & Howell, 1956; Pine, Howell & Watson, 1960). Unlike the *Actinomyces*, but similar to the glucose fermentations of species of *Propionibacterium*, *Actinomyces propionicus* fermented glucose with the formation of CO₂, acetic and propionic acids, and small amounts of formic, lactic and succinic acids. Thus unlike the *Actinomyces*, CO₂ was not required for anaerobic growth but was a major product of fermentation. Like the fermentations of *Propionibacterium* (Wood *et al.* 1937) significant amounts of lactic acid were not consistently formed, whereas this is a major product of all *Actinomyces* fermentations. Like *A. naeslundii* (Buchanan & Pine, unpublished

results) and *P. arabinosum* (Ichikawa, 1957), *A. propionicus* was facultative and fermented glucose in the presence of oxygen to form stoichiometric amounts of CO₂ and acetic acid. However, unlike *A. naeshlundii*, *A. propionicus* did not require CO₂ to grow and ferment glucose in the presence of oxygen.

Table 7. *A suggested phylogenetic progression in the order Actinomycetales*

Organism	Characters
<i>Lactobacillus bifidus</i>	Branching cells, no true mycelium. Anaerobic, catalase negative. Ferments glucose with formation of large amounts of lactic (L+) and acetic acid, traces of formic and succinic also formed. Lysine in cell wall. Parasitic (Norris, Flanders, Tomarelli & György, 1950; Frank & Skinner, 1954; Pine & Howell, 1956; Cummins, Glendenning & Harris, 1957).
<i>Actinomyces israelii</i>	Branching cells, true mycelium formed. Anaerobic, catalase negative. Ferments glucose and CO ₂ to form acetic, formic, lactic (L+), and succinic acids. Lysine in cell wall. Parasitic and pathogenic. (Pine & Howell, 1956; Cummins & Harris, 1958).
<i>A. naeshlundii</i>	Branching cells, true mycelium formed. Facultative, catalase negative. Ferments glucose and CO ₂ to form lactic (L+), formic, acetic and succinic acids. Aerobically, glucose is oxidized in the presence of CO ₂ to form CO ₂ and acetic acid. Lysine in cell wall. Parasitic and pathogenic (Pine & Howell, 1956; Cummins & Harris, 1958; Howell <i>et al.</i> 1959; Buchanan & Pine, unpublished results).
<i>A. propionicus</i>	Branching cells, true mycelium formed. Facultative, catalase negative. Ferments glucose to form CO ₂ , acetic and propionic acids with small amounts of lactic (DL) and succinic acids. Aerobically, glucose is oxidized to form CO ₂ and acetic acid. Diaminopimelic acid in cell wall. Parasitic and pathogenic.
X (<i>Propionibacterium</i>) species	Branching cells, true mycelium not formed. Facultative, catalase positive. Ferments glucose to form CO ₂ , acetic, succinic and propionic acids. Lactic acid fermented. Aerobically, glucose is oxidized to form CO ₂ and acetic acid. Diaminopimelic acid in cell wall. Enzymes oxidizing citric acid cycle intermediates present. Saprophytic (Hitchner, 1934; Wood & Werkman, 1936; Delwiche & Carson, 1953; Ichikawa, 1957; Cummins & Harris, 1958).
Y (<i>Corynebacterium</i>) species	Branching cells, true mycelium not formed. Facultative to aerobic, catalase positive, cytochrome transport system present. Ferments glucose in decreased oxygen supply to form CO ₂ , acetic, formic, lactic, succinic, and propionic acids. With adequate oxygen, these products oxidized to CO ₂ and H ₂ O. Diaminopimelic* acid in cell wall. Parasitic, pathogenic, saprophytic (Tasman & Brandwijk, 1940; Pappenheimer & Hendee, 1947; Cummins & Harris, 1956).
<i>Bacterionema matruchotii</i> (<i>Leptotrichia dentium</i>)	Branching cells, true mycelium formed. Facultative to strict aerobes, catalase positive. Ferments glucose in decreased oxygen supply to form CO ₂ , formic, acetic, propionic, lactic (DL) and succinic acids. With adequate oxygen, glucose and lactic acid oxidized presumably to CO ₂ and water. Diaminopimelic acid present in cell wall. Parasitic. (Davis & Baird-Parker, 1959; Gilmour, 1961; Pine & Howell, 1961).
<i>Nocardia</i> and <i>Streptomyces</i> species	Branching cells, true mycelium formed with production of chains of endospores. Strict aerobes, glucose and various carbohydrates oxidized to CO ₂ and H ₂ O. Catalase positive. Diaminopimelic acid in cell wall. Saprophytic and pathogenic (Gordon & Mihm, 1958; Gilmour, Butterworth, Noble & Wang, 1955; Butterworth, Gilmour & Wang, 1955; Cummins & Harris, 1958; Moore & Chapman, 1959).

* Of ten species of *Corynebacterium* studied by Cummins & Harris (1956), two (*C. pyrogenes* and *C. hemolyticum*) had lysine instead of diaminopimelic acid.

Hungate (1946) described a propionic acid fermentation by the strict anaerobe *Micromonospora propionici* (*Actinobacterium propionici*, Prévot, 1957) isolated from the gut of a wood termite. Although its anaerobic fermentations were identical with those of *Actinomyces propionicus* and its morphology similar, it would appear that *M. propionici* is a different organism from *A. propionicus* on the basis of habitat, anaerobic requirements and use of cellulose as a substrate.

The form of lactic acid formed by a species has been used for taxonomic differentiation particularly among the members of the family Lactobacteriaceae. There has been no report on the kind of lactic acid formed by strains of *Propionibacterium*. However of the *Actinomyces* spp. studied, *A. naeslundii*, *A. israelii*, and *A. bovis* form L(+)-lactic acid. On the other hand, *A. propionicus* was found to produce DL-lactic acid, as did *Bacterionema matruchotii*, which is a genus considered phylogenetically intermediate between *Actinomyces* and *Nocardia* (Gilmour *et al.* 1961; Gilmour 1961; Gilmour & Beck, 1961; Howell & Pine, 1961). This observation serves to relate these two species (Table 7) as does their common ability to form propionic acid (Howell & Pine, 1961) and similar cell-wall constituents (Davis & Baird-Parker, 1959).

Cummins & Harris (1956, 1958) and Davis & Baird-Parker (1959) have emphasized the taxonomic value of cell-wall composition to determine species and generic relationships among members of the actinomycetes. In this regard, cell walls of *Actinomyces propionicus* were found to contain glucose, galactose, small amounts of glucosamine and galactosamine, and aspartic and diaminopimelic acids. This composition is similar to that of species of *Propionibacterium* except the latter had glycine and no aspartic acid (Cummins & Harris, 1956, 1958). The cell-wall composition of *A. propionicus* was essentially the same as that of *Bacterionema matruchotii* (*Leptotrichia dentium*) except the walls of the latter contained no galactose or aspartic acid. *A. propionicus* differed from all other members of the genus *Actinomyces* in the sugars found in the cell wall and in the fact that all other *Actinomyces* species had lysine instead of diaminopimelic acid (Cummins & Harris, 1958, 1959). Therefore, on the basis of cell-wall composition *A. propionicus* is distinct from all related organisms.

It is apparent that the interest in *Actinomyces propionicus* lies in its phylogenetic relationship to other actinomycetes and to propionibacteria. Starting with the simple branching anaerobic *Lactobacillus bifidus* var. *pennsylvanicus* (Sundman, Björkstén & Gyllenberg, 1959), one may make a morphological and physiological progression to the more complex sporulating actinomycetes having an aerobic metabolism (Table 7). Table 7 shows the relationships of members of the genus *Actinomyces* to some other organisms which have been classified in the Actinomycetales, or which have been recognized by various investigators as being related to members of this order. So far there have been described no species forming true mycelium which might fill the gaps X and Y in Table 7, and which might have the progressively more complicated metabolic characteristics found in the genera *Propionibacterium* and *Corynebacterium*. Although the relationships of members of these genera to members in the genus *Actinomyces* have long been recognized on a morphological and physiological basis (Bergey's *Manual*, 1934, 1939; Stanier & Van Niel, 1941), these taxonomic schemes have been complicated by the assignment of a more complex cellular structure to *Actinomyces*, because of the formation

of a true mycelium. Consequently on a morphological basis, *Propionibacterium* and *Corynebacterium* have been considered to precede the more complex *Actinomyces* (Stanier & Van Niel, 1941; Prévot, 1957).

However, in the light of present knowledge about the physiological basis of morphology and its relationship to cell-wall composition (Duguid & Wilkinson, 1961; Glick, Sall, Zilliken & Mudd, 1960), the formation of branching cells with true hyphal elements may be considered the result of a decrease, rather than an increase, in enzymic complexity. Formation of buds or branches due to defective cell-wall synthesis and the formation of branched hyphae and even mycelium due to delayed cross-wall formation might represent an impaired operation of cell-wall synthesizing enzymes. This functional loss need not necessarily benefit the organism nor be conducive to its survival. On the other hand, the formation of chains of catenulae (oidia) observed in strains of *Nocardia* or *Streptomyces* (Gordon & Mihm, 1958; Moore & Chapman, 1959) most certainly indicates a more complex morphological development pertinent to the life cycle and increased survival of the organism. From these considerations it is not unreasonable to substitute *Propionibacterium* for X in Table 7 as a metabolically more complex genus following *Actinomyces* (*A. propionicus*), and *Corynebacterium* for Y in the postulated phylogenetic progression from *Lactobacillus* to *Streptomyces* shown in Table 7. Further clarification of the taxonomic relationships of these genera with more recently described genera and species such as *Bacterionema matruchotii* and *Nocardia salivae* will require additional information about the intermediary metabolism of these organisms and members of the genus *Actinomyces*.

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EXPLANATION OF PLATES

PLATE 1

Cellular morphology of *Actinomyces propionicus*

Fig. 1. Typical branching cells grown anaerobically for 72 hr. on 5 ml. of 1 % (w/v) glucose + Casitone medium. Gram stain, $\times 970$.

Fig. 2. Long filamentous forms grown anaerobically for 72 hr. in 1 l. fermentation flasks of 0.5 % (w/v) glucose + Casitone medium. Gram stain, $\times 970$.

Fig. 3. Typical organisms grown anaerobically for 10 days in 5 ml. of 1 % (w/v) glucose + Casitone medium showing terminal formation of spheroplasts. Methylene blue wet mount, $\times 970$.

Fig. 4. Cells grown for 10 days on 5 ml. of 1 % (w/v) raffinose + Casitone medium showing formation of greatly enlarged spheroplasts. Methylene blue wet mount, $\times 970$.

Fig. 5. Pus from lesion in mouse inoculated with *A. propionicus*. Mouse was killed after 33 days. Gram stain, $\times 970$.

PLATE 2

Comparison of colony morphology of *Actinomyces propionicus* and *Propionibacterium arabinosum*

Fig. 6. Colonies of *A. propionicus* grown 24 hr. on 1 % (w/v) glucose + Casitone agar. $\times 100$.

Fig. 7. Colonies of *P. arabinosum* grown 24 hr. on 1 % (w/v) glucose + Casitone agar. $\times 100$.

Fig. 8. Colonies of *A. propionicus* grown 24 hr. on 1 % (w/v) glucose + Casitone agar. $\times 430$.

Fig. 9. Colonies of *P. arabinosum* grown 24 hr. on 1 % (w/v) glucose + Casitone agar. $\times 430$.