DAVIS, G. H. G. & BAIRD-PARKER, A. C. (1959). *J. gen. Microbiol.* 21, 612-621

The Classification of Certain Filamentous Bacteria with Respect to their Chemical Composition

BY G. H. G. DAVIS AND A. C. BAIRD-PARKER

Bacteriology Department, University of Birmingham

SUMMARY: Upon the basis of cell-wall composition the oral bacteria *Leptotrichia dentium* and *L. buccalis* appear to form a natural generic group related to *Nocardia* and possibly *Lactobacillus*. An aerobic oral actinomycete of the type commonly called *Nocardia* has been shown to resemble closely the anaerobic pathogenic *Actinomyces* which parasitizes man. The cell-wall composition of *Streptomyces*, *Nocardia*, *Lactobacillus* and *Fusobacterium* strains is also reported.

The purpose of this study is to determine the relationship between the filamentous bacteria of the oral flora and between these and the comparable forms from other sources.

The oral parasite, *Leptotrichia dentium* (Baird-Parker & Davis, 1958; Davis & Baird-Parker, 1959) possesses characters that suggest possible relationship with both *Nocardia* and *Streptomyces*. Nevertheless, it does not resemble either of these genera so closely as to make classification with one of them unequivocal upon morphological grounds alone. At the same time, the diagnostic characters of *Streptomyces* and *Nocardia* overlap to some extent; some species of the latter, e.g. *N. asteroides*, produce aerial mycelium and spores of distinctly *Streptomyces*-like appearance (Gordon & Mihm, 1958) and the vegetative mycelium of *Streptomyces* commonly fragments to yield *Nocardia*-like elements (Bisset, 1957; Bradley & Anderson, 1958).

It has, on the other hand, been suggested that *Leptotrichia buccalis* is closely related to *Lactobacillus* upon metabolic grounds (Hamilton & Zahler, 1957).

Although *Leptotrichia dentium* more closely resembles the Actinomycetales in its morphology, it is physiologically similar to *L. buccalis* in many ways and partly for this reason, and partly for convenience, the two organisms currently constitute the ill-defined genus *Leptotrichia*.

Leptotrichia buccalis has also been frequently confused with *Fusobacterium* so that certain specific names in this genus [e.g. *F. dentium* (Hine & Berry, 1937); *F. plauti-vincenti* (Jackins & Barker, 1951)] are synonyms for *L. buccalis*.

It is apparent that the relationships of all these genera and species require to be re-examined.

A further problem is the occurrence in the mouth of aerobic Gram-positive filamentous bacteria usually classified as *Nocardia* spp. (Morris, 1954). Although some of these have been identified with previously described species, e.g. *Nocardia corallina*, *N. rangoonensis*, *N. mesenterica*, by Mandel, Levy & Wasserman (1957) their taxonomy has not been developed to any great extent. Such organisms have been confused with the microaerophilic *Actinomyces israelii* and it is probable that the *A. naeslundii* of Thompson & Lovestedt (1951) and

Garrod (1952) represents one of the commonest of these oral organisms. The group 3b of Onisi & Nuckolls (1958) also appears to consist of such organisms.

We have attempted to resolve some of these problems by supplementing cultural, morphological and metabolic studies with chromatographic analyses of cell walls according to the technique of Cummins & Harris (1956, 1958).

MATERIALS AND METHODS

See Table 1 for strains used and their origins. The soil strains of *Nocardia* were supplied by Dr R. B. Cain of this department.

The isolation and maintenance of *Leptotrichia dentium* and *L. buccalis* has been described elsewhere (Davis & Baird-Parker, 1959) and so also has the isolation and classification of *Lactobacillus* spp. (Davis, 1955), and the isolation of *Fusobacterium* spp. (Baird-Parker, 1957).

Table 1. *Strains examined*

Strain no.	Description	Source
SG	<i>Streptomyces griseus</i>	Old laboratory culture
S25	<i>Streptomyces</i> sp.	Soil
S26	<i>Streptomyces</i> sp.	Soil
600	<i>S. hortensis</i>	NCTC
4523	<i>Actinomyces buccalis</i>	NCTC
8595	<i>Nocardia asteroides</i>	NCTC
C1	<i>Nocardia</i> sp.	Soil
C2	<i>Nocardia</i> sp.	Soil
2A1	<i>Leptotrichia dentium</i>	Human saliva
1C1	<i>L. dentium</i>	Human saliva
V1	<i>L. dentium</i>	Human saliva
LD	<i>L. dentium</i>	Human saliva
LB1	<i>Leptotrichia buccalis</i>	Human saliva
LB2	<i>L. buccalis</i>	Human saliva
LB3	<i>L. buccalis</i>	Human saliva
G5A	Aerobic actinomycete	Human saliva
T3	Aerobic actinomycete	Human saliva
4A	<i>Lactobacillus fermenti</i>	Human saliva
6G	<i>L. buchneri</i>	Human saliva
3D	<i>L. plantarum</i>	Human saliva
2A	<i>L. casei</i>	Human saliva
8C	<i>L. salivarius</i>	Human saliva
1A	<i>L. acidophilus</i>	Human saliva
F18	<i>Fusobacterium</i> sp.	Human saliva
FP	<i>Fusobacterium</i> sp.	Human saliva

Oral aerobic actinomycetes were isolated upon nutrient agar supplemented with 0.3% (w/v) yeast extract and 0.2% (w/v) soluble starch. This medium was also used for the maintenance of *Streptomyces*, *Nocardia*, and the NCTC strains.

Chromatographic analysis

Cell growth. All strains were grown in liquid media in bulk cultures. The aerobic organisms, i.e. *Nocardia*, *Streptomyces*, *Leptotrichia dentium*, oral aerobic actinomycetes, and NCTC strains were inoculated into Carrel type flasks containing 200 ml. of nutrient broth plus 1% (w/v) glucose and 0.2%

(w/v) soluble starch, pH 7.4, in shallow layers. Soil bacteria were incubated at 28°, the rest at 37°, and the flasks shaken daily to distribute growth. Cells were harvested when good growth was evident, usually 3–5 days.

Lactobacillus strains were cultured in screw-capped bottles containing the following medium: vitamin-free casein hydrolysate 20 g., yeast-extract 5 g., sodium acetate 15 g., glucose 20 g., salts A 5 ml., salts B 5 ml., Tween 80 1 ml., distilled water 1000 ml., pH 7.0. (For salts A and B see Hayward, 1957.)

Inoculated bottles of medium were loosely closed, especially in the case of heterofermentative strains, to allow generated gas to escape.

Anaerobic organisms (*Leptotrichia buccalis* and *Fusobacterium*) were grown in tightly closed screw-capped bottles completely filled with Baird-Parker's (1957) medium, omitting the antibiotics and dye.

Cells were harvested by centrifuging, and washed three times in distilled water. (In some cases, e.g. *Nocardia* and *Streptomyces*, the alkaline ethanol treatment of Cummins & Harris (1958) was applied.) The cell mass was divided into two parts with normally the harvest of 200 ml. of medium in each.

Sugar extracts. One sample of cells was suspended in *c.* 5 ml. of 2N-H₂SO₄ in a tightly closed 20 ml. screw-capped McCartney bottle, and heated for 4 hr. in a steamer. This whole cell hydrolysate was neutralized to pH 4.8 using freshly prepared saturated barium hydroxide solution and methyl red as indicator. Precipitated sulphate and cells were removed by centrifuging and the clear supernatant evaporated to dryness in vacuum over calcium chloride. The extract was re-suspended in *c.* 0.2–0.5 ml. 10% iso-propyl alcohol solution.

Amino acid extracts. The second sample of cells was made into a thick slurry with water and the cells broken by exposing them to high-frequency vibrations using a 'Mullard' 60 W. ultrasonic drill fed by an amplifier, supplying 180 W. at 20,000 kc./s.

The rate of cell breakage by this method varies greatly with different types of organism. Gram-negative organisms (*Fusobacterium*, *Escherichia coli*) break readily (1–2 min. exposure); Gram-positive filamentous bacteria (*Streptomyces*, *Corynebacterium*, etc.) require 20–30 min.; small Gram-positive bacilli (certain lactobacilli) and coccal forms are difficult to rupture, requiring 1 hr. or more. Broken cells were centrifuged, resuspended in 0.5% trypsin solution, and solid sodium bicarbonate added to produce *c.* pH 8.6. trypsin digestion was carried out at 37° for 12–18 hr.; the cell walls were centrifuged, washed three times in distilled water, and resuspended in *c.* 5 ml. 6N-HCl. Hydrolysis was carried out in tightly closed screw-capped McCartney bottles by autoclaving at 25–30 lb./sq.in. pressure for 4 hr. The hydrolysate was filtered through sintered glass (porosity no. 4), evaporated to dryness on a steam bath, re-dissolved in 1–2 ml. 10% iso-propyl alcohol, one spot of concentrated ammonia added and the extract refiltered, evaporated dry and re-dissolved in *c.* 0.3 ml. 10% iso-propyl alcohol.

Chromatography. Amino acid and sugar extracts were analysed by paper chromatography using the descending, one-dimensional method, and Whatman no. 1 paper.

Each extract was run in two solvents.

Sugars: (a) Iso-propyl alcohol (160) + water (40).

(b) Phenol (80) + water (20).

Amino acids: (a) Iso-propyl alcohol (70) + acetic acid (10) + water (20).

(b) Phenol (80) + water (20) + 8 hydroxyquinoline (0.008 g./100 ml.) in an atmosphere of ammonia.

Owing to the varying concentrations of different extracts, it was found advantageous to run each extract at three or four concentrations upon the chromatogram. This was achieved by using a platinum loop to load the chromatogram, applying, for example, 2, 6 and 10 spots while gently heating with a hair-dryer to control the spot size.

Sugar chromatograms were air-dried, dipped in aniline hydrogen phthalate and heated at 105° C. for 5 min.

Amino acid chromatograms were sprayed with 0.25% (w/v) ninhydrin in acetone and heated at 105° C. for 5 min.

The isomers of diaminopimelic acid were differentiated, as far as this is possible, using Hoare & Work's (1957) method.

Biochemical and cultural characters

It is commonly stated that biochemical and cultural characters are of little value in differentiating Gram-positive filamentous bacteria into definite groups. In view of the small numbers of strains here described we would not dispute this statement, but simply record the results of certain tests which seem to us to be of possible value in the characterization of the aerobic types we have studied (Table 2). The differentiation of *Leptotrichia buccalis* from *L. dentium* has been described (Davis & Baird-Parker, 1959) and the characteristics of *Fusobacterium* spp. will form the basis of a subsequent publication.

Casein digestion and Xanthine and Tyrosine dissolution tests were carried out as described by Gordon & Mihm (1956). Gelatin liquefaction was tested by the method of Frazier (1926). Routine methods were used for the remaining tests.

RESULTS

The results are set out in Tables 2-4. In recording the cell-wall patterns we have adopted an arbitrary method whereby the major components are scored as positives and the minor components as traces. Using the relatively simple chromatographic methods here described it is considered safest to allow taxonomic significance only to major components, and as can be seen in Table 3 this renders the classification of organisms into groups at generic level quite easy. Seven such groups were differentiated and their salient cell-wall characteristics are compared in Table 4. The analyses obtained for *Streptomyces*, *Nocardia* and *Lactobacillus* spp. agree in the main with those reported by Cummins & Harris (1956, 1958). It will be noted that most of the bacteria yield ribose upon sugar analysis; this is to be expected in view of the presence of ribonucleic acid in bacteria; it is unlikely, however, that any other sugar is

Table 2. *Selected physiological and cultural characters of various aerobic actinomycetes*

Strain	No. of strains	Catalase production	Nitrate reduction	Nitrite reduction	Gelatin liquefaction	Starch hydrolysis	Casein digestion	Hippurate hydrolysis	Tyrosine dissolution	Xanthine dissolution	Final pH in 1 % glucose broth	Growth temperature	Growth in glucose broth			
													flocculent	granular	granular	smooth turbidity
Soil streptomycetes	3	+	+	—	+	+	+	—	W	—	7.5	28°				
<i>Streptomyces horttonensis</i>	1	+	+	—	+	+	W	—	—	—	7.5	better at 28°				
NCTC 600																
<i>Actinomyces buccalis</i>	1	+	—	—	+	+	+	—	+	W	7.5	28 or 37°				
NCTC 4523																
Soil Nocardia	3	+	—	—	—	W	—	W	+	—	8.3	28°				
<i>Nocardia asteroides</i>	1	+	+	—	—	W	—	—	—	—	8.0	37°				
<i>Leptotrichia dentium</i>	4	+	+	—	—	+	—	+	—	—	5.2	37°				
Aerobic oral actinomycetes	3	+	+	+	+	W	—	—	—	—	5.2	37°				

+ = positive, W = weakly positive, — = negative.

Table 3. Cell-wall composition of strains studied

Group	Strain	Unidentified hexosamine	Rhamnose	Galactose	Glucose	Ribose	Arabinose	Slow runner	Aspartic acid	Leucine	Serine	Glycine	Alanine	Glutamic acid	Lysine	DL-or DD-Diamino-pimelic acid	LL-Diamino-pimelic acid
Streptomyces	<i>S. griseus</i> S25	Tr.			Tr.	Tr.				Tr.	Tr.	+	+	+	Tr.		+
	S26				Tr.				Tr.			+	+	+			+
	NCTC 4523											Tr.	Tr.	+		+	
	NCTC 600											Tr.	Tr.	+		+	
Nocardia	C1				+		+			Tr.		Tr.	+	+		+	
	C2				+		+			Tr.		Tr.	+	+		+	
<i>Leptotrichia dentium</i>	NCTC 8595											Tr.	+	+		+	
	2A1											Tr.	+	+		+	
	1C1											Tr.	+	+		+	
	V1											Tr.	+	+		+	
<i>Leptotrichia buccalis</i>	LD											Tr.	+	+		+	
	LB1											Tr.	+	+		+	
	LB2											Tr.	+	+		+	
Oral aerobic actinomycetes	LB3											Tr.	+	+		+	
	G5A											Tr.	+	+		+	
	T3											Tr.	+	+		+	
Lactobacillus	<i>L. fermenti</i>											Tr.	+	+		+	
	<i>L. buchneri</i>											Tr.	+	+		+	
	<i>L. salivarius</i>											Tr.	+	+		+	
	<i>L. acidophilus</i>											Tr.	+	+		+	
Fusobacterium	<i>L. casei</i>											Tr.	+	+		+	
	<i>L. plantarum</i>											Tr.	+	+		+	
	F18											Tr.	+	+		+	
	FP											Tr.	+	+		+	

+ = major component, Tr. = minor component, - = not detected.

Table 4. *Comparative cell-wall patterns of certain groups and genera of bacteria*

	LL-Di- amino- pimelic acid	DL or DD- Diamino- pimelic acid	Lysine	Glutamic acid	Alanine	Glycine	Serine	Leucine	Aspartic acid	Slow runner	Arabi- nose	Glucose	Galac- tose
<i>Streptomyces</i>	+	-	-	+	+	+	-	-	-	-	-	-	-
<i>Nocardia</i>	-	+	-	+	+	-	-	-	-	-	+	-	+
<i>Leptotrichia dentium</i>	-	+	-	+	+	-	-	-	-	-	+	+	-
<i>L. buccalis</i>	-	+	-	+	+	-	-	-	-	-	-	+	-
Oral aerobic actinomycetes	-	-	+	+	+	-	-	-	-	-	-	-	+
<i>Lactobacillus</i>	No set	generic	pattern										
<i>Fusobacterium</i>	-	-	+	+	+	+	+	+	+	+	-	+	-
<i>Actinomyces israelii</i> *	-	-	+	+	+	-	-	-	-	-	-	-	+

* From Cummins & Harris, 1958.

commonly present in large amounts in the cytoplasm or nucleus and the non-ribose sugars detected in whole cell hydrolysates may be assumed to be cell-wall components. We have compared sugar analyses of whole cells and cell walls in a few representative strains and found them to be identical.

Amino sugars, which appear to occur almost universally in bacterial cell walls, were not readily detected by the methods here described. They could not be picked out upon the amino acid chromatograms, but were often noticeable in the sugar runs. However, the latter method does not allow their clear differentiation in most cases and spots representing glucosamine, galactosamine or muramic acid are therefore recorded under a common heading as hexosamines (Table 3).

DISCUSSION

The classification of bacteria must depend upon information obtained by the use of various techniques. Chemical analysis of bacterial cellular components appears to be a useful method of confirming taxonomic groupings and in some cases clarifying relationships, which may be otherwise obscure, between certain bacteria.

In the present study we have tried to keep the technical work within the scope of the non-specialist and extract the maximum of useful information by the simplest means, hence the use of whole cell hydrolysates for sugar analyses and one-dimensional chromatography. The questions posed in the introduction to this paper may be answered as follows:

Leptotrichia dentium resembles the genus *Nocardia* in cell-wall amino-acid pattern, but differs from it in sugar pattern. No relationship with the genus *Streptomyces* is indicated.

Leptotrichia dentium and *L. buccalis* exhibit the same amino acid patterns but differ by the absence of arabinose in the sugar component of *L. buccalis*. Whether these results indicate that the two organisms may be regarded without doubt as two species of a single genus depends upon the taxonomic significance placed upon the amino-acid and sugar patterns respectively. It has been suggested that the amino-acid patterns characterize genera and sugar patterns species, but too little evidence is yet available to prove this idea. In the *Nocardia-Corynebacterium-Mycobacterium* group of genera there appears to be little variation in either pattern between different species or even genera (Cummins & Harris, 1958). Although lacking final proof it therefore seems reasonable to retain the genus *Leptotrichia* as distinct from, but closely related to, the genus *Nocardia* and to retain *L. dentium* and *L. buccalis* as its two species.

Among the lactobacilli, *Lactobacillus plantarum* and *L. casei* show cell-wall patterns grossly similar to *Leptotrichia buccalis* in both amino acids and sugars. In contrast to such groups as *Nocardia-Mycobacterium* referred to above, there is considerable variation of pattern between species of the genus *Lactobacillus* and upon the basis of cell-wall pattern and physiology the inclusion of *Leptotrichia buccalis* as a species of the genus *Lactobacillus* would not appear anomalous. Morphologically, however, no *Lactobacillus* sp. resembles the distinctive *Leptotrichia buccalis* form, nor is it feasible to place *L. dentium*

with the lactobacilli on morphological or physiological grounds. The question thus resolves into the choice between placing *L. buccalis* in the genus *Lactobacillus* and *Leptotrichia dentium* in the genus *Nocardia*, with neither grouping being perfect, or retaining the genus *Leptotrichia* as suggested above. *Fusobacterium* spp. have a cell-wall pattern distinguished by its relative complexity and the presence of a distinctive slow running component of unknown composition. Amino acid complexity is characteristic of the cell walls of Gram-negative species. The slow running component runs slower than diaminopimelic acid in the iso-propyl alcohol solvent, and in Hoare & Work's solvent it can be separated into two slow running spots.

The *Nocardia*-like aerobic actinomycetes isolated from the human mouth for this study are not classifiable as members of the genus *Nocardia* on the evidence of cell-wall patterns. They lack both diaminopimelic acid and arabinose, and upon the results so far available appear to have a cell-wall composition very like the true, microaerophilic *Actinomyces* of human origin (Cummins & Harris, 1958). If this classification should prove correct the definition of the genus *Actinomyces* will require modification to include aerobic non-pathogenic organisms of this type.

It is interesting to note that the soil *Nocardia* strains are identical in cell-wall composition with *Nocardia asteroides*, which is morphologically quite different from them in that it produces aerial chains of spores. The only *Nocardia* spp. investigated by Cummins & Harris (1958) which was not of animal origin, was *N. gardneri* and this turned out to be a *Streptomyces*. Although *Nocardia* is predominantly a saprophytic genus, the atypical animal strains are commonly used as examples of the group; our results show that *Nocardia* spp. from saprophytic and parasitic sites, differing widely in morphological potential, nevertheless form a cohesive group in terms of cell-wall composition.

Upon biochemical as well as cell-wall characters the NCTC strain 4523 called *Actinomyces buccalis* should be re-classified with *Streptomyces*.

REFERENCES

- BAIRD-PARKER, A. C. (1957). Isolation of *Leptotrichia buccalis* and *Fusobacterium* species from oral material. *Nature, Lond.* **180**, 1056.
- BAIRD-PARKER, A. C. & DAVIS, G. H. G. (1958). The morphology of *Leptotrichia* species. *J. gen. Microbiol.* **19**, 446.
- BISSET, K. A. (1957). Some observations upon the mode of sporulation and relationships of monosporous Actinomycetes. *J. gen. Microbiol.* **17**, 562.
- BRADLEY, S. G. & ANDERSON, D. L. (1958). Comparative study on *Nocardia* and *Streptomyces*. *Bact. Proc.* p. 49.
- CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell walls in some Gram-positive bacteria and its possible value as a taxonomic character. *J. gen. Microbiol.* **14**, 583.
- CUMMINS, C. S. & HARRIS, H. (1958). Studies on the cell-wall composition and taxonomy of Actinomycetales and related groups. *J. gen. Microbiol.* **18**, 173.
- DAVIS, G. H. G. (1955). The classification of Lactobacilli from the human mouth. *J. gen. Microbiol.* **13**, 481.

- DAVIS, G. H. G. & BAIRD-PARKER, A. C. (1959). *Leptotrichia buccalis*. *Brit. dent. J.* **106**, 70.
- FRAZIER, W. C. (1926). A method for the detection of change in gelatin due to bacteria. *J. infect. Dis.* **39**, 302.
- GARROD, L. P. (1952). Actinomycosis of the lung: aetiology, diagnosis and chemotherapy. *Tubercle*, **33**, 258.
- GORDON, R. E. & MIHM, J. M. (1957). Comparative study of some strains received as Nocardiae. *J. Bact.* **73**, 15.
- GORDON, R. E. & MIHM, J. M. (1958). Sporulation by two strains of *Nocardia asteroides*. *J. Bact.* **75**, 239.
- HAMILTON, R. D. & ZÄHLER, S. A. (1957). A study of *Leptotrichia buccalis*. *J. Bact.* **73**, 886.
- HAYWARD, A. C. (1957). Detection of gas production from glucose by heterofermentative lactic acid bacteria. *J. gen. Microbiol.* **16**, 9.
- HINE, M. K. & BERRY, G. P. (1937). Morphological and cultural studies of the genus *Fusiformis*. *J. Bact.* **34**, 517.
- HOARE, D. S. & WORK, E. (1957). The stereoisomers of α - ϵ -diaminopimelic acid. 2. Their distribution in the bacterial order Actinomycetales and in certain Eubacteriales. *Biochem. J.* **65**, 441.
- JACKINS, H. C. & BARKER, H. A. (1951). Fermentation processes of the fusiform bacteria. *J. Bact.* **61**, 101.
- MANDEL, I. D., LEVY, B. M. & WASSERMAN, B. H. (1957). Histochemistry of calculus formation. *J. Periodont.* **28**, 132.
- MORRIS, E. O. (1954). The bacteriology of the oral cavity. V. *Corynebacterium* and Gram-positive filamentous organisms. *Brit. dent. J.* **97**, 29.
- ONISI, M. & NUCKOLLS, J. (1958). Descriptions of actinomycetes and other pleomorphic organisms recovered from pigmented carious lesions of the dentine of human teeth. *Oral Surg.* **11**, 910.
- THOMPSON, L. & LOVESTEDT, S. A. (1951). An actinomyces-like organism obtained from the human mouth. *Proc. Mayo Clin.* **26**, 169.

(Received 30 April 1959)