A Study of the Overall Similarity of Certain Actinomycetes Mainly of Oral Origin

By T. H. MELVILLE

School of Dental Surgery, University of Liverpool

(Received 29 December 1964)

SUMMARY

Computer techniques were applied to the study of 71 isolates of branching or diphtheroid bacteria from human and bovine sources. Features including morphological and colonial characteristics, nutritional and environmental growth requirements, fermentative abilities and end-products and cell-wall composition were used to determine the relationships between these organisms. The features were determined with aerobic or anaerobic growth conditions or both, according to the nature of the individual bacteria. Relationships between facultatively anaerobic micro-organisms were very similar whether the features were determined aerobically or anaerobically; some apparently close relationships were shown between preferentially aerobic and preferentially anaerobic bacteria when they were compared with facultatively anaerobic micro-organisms.

The analysis separated groups of organisms corresponding to *Actinomyces naeslundii*, *A. bovis*, *Nocardia salivae*, *Bacterionema matruchotii* (*Leptotrichia dentium*) and *Corynebacterium* spp. The members of the various genera showed little affinity to each other. *A. naeslundii* and *A. bovis* were fairly closely related and some unplaced isolates, including typical *A. israelii* strains, showed a lesser relationship to either or both of these species. Isolates of *A. odontolyticus* showed high similarity to *A. bovis* and were included in this species despite their better aerobic growth. Several isolates of *A. naeslundii* grew well aerobically and produced catalase; production of this enzyme is not, by itself, sufficient to remove an organism from the genus *Actinomyces*.

INTRODUCTION

In the study of actinomycosis there have been many disagreements about the nomenclature and taxonomy of the bacteria involved and the identity or otherwise of strains of human and animal origin. The identification and naming of bacteria of this general description found in dental plaques and in carious cavities, has been even more controversial. Part of these difficulties has been due to the changing state of knowledge over the years and part to the varied emphasis placed on certain characteristics as taxonomic criteria by individual taxonomists. The historical background to these problems may be deduced from the monograph by Buchanan (1925) and is dealt with in greater detail elsewhere (Melville, 1964). A wide selection of organisms must be chosen for a classification by overall similarity so that boundary lines are not predetermined, but there are practical limits to the numbers of isolates which can be dealt with at the one time. The present investigation has been confined to parasitic or pathogenic branching or diphtheroid organisms from the human oral

cavity with some bovine oral strains for comparative purposes. It must be borne in mind that all taxonomic conclusions drawn are subject to the proviso that this group of organisms was selected by oral origin.

METHODS

Organisms. The 71 isolates used in this study are listed in Table 1 with their source and the name under which they were received.

Features. Each of the isolates was characterized by using the following criteria: colonial and cellular morphology of well isolated 5-day colonies grown at 37° on brain heart agar plates; microcolony formation examined as soon as growth was evident; cell wall composition (Cummins & Harris, 1956); ability to grow on certain media, in the presence of various inhibitory factors and under various environmental conditions (Table 2); biochemical features (Table 3). Full details of the methods used are given elsewhere (Melville, 1964).

Table 1. *Source of organisms used*

Organisms	Received as	Source
3 strains	<i>A. naeslundii</i>	Dr A. Howell Jun., Nat. Inst. Dent. Res., Bethesda, Maryland
8 strains	<i>A. bovis</i>	Dr L. Pine, Duke University, N. Carolina
2 strains	<i>A. bovis</i>	NCTC 9429 and 4500
2 strains	<i>A. israelii</i>	Dr A. Howell Jun.
4 strains	<i>A. israelii</i>	Dr J. W. Porteous, Marishal College, Aberdeen
2 strains	<i>A. israelii</i>	NCTC 8047 and 6880
1 strain	<i>A. propionicus</i>	ATCC 14157*
2 strains	<i>A. odontolyticus</i>	NCTC 9981 and 9985*
1 strain	<i>N. salivae</i>	NCTC 10207*
1 strain	<i>B. matruchotii</i>	Dr Marion Gilmour, Eastman Dental Dispensary, Rochester, N.Y.
1 strain	<i>Leptotrichia dentium</i>	NCTC 10206*
7 strains	—	Swabs from patients suspected of having actinomycosis. One from womb, rest oral
14 strains	—	Swabs from patients not suspected of having actinomycosis. Oral origin
5 strains	—	Root canal samples from endodontic cases
2 strains	—	Gingivae in normal mouths
3 strains	—	Tooth surfaces in normal mouths
11 strains	—	Saliva from normal mouths
1 strain	<i>C. diphtheriae</i>	Old laboratory stock culture
1 strain	<i>Lactobacillus</i> sp.	Saliva from normal mouth

* Original type strains.

The characters of the organisms compared in overall similarity studies are phenetic, the results of testing the organisms under set conditions. When features were studied in liquid or semi-solid media the aerobes and anaerobes could grow and demonstrate their characteristics at the appropriate site in the bottle. Cultures on solid media were grown aerobically, or anaerobically in nitrogen + 5% (v/v) CO₂. These tests were duplicated for organisms capable of good growth both aerobically and anaerobically.

Certain of the features observed proved to be of no differential value and did not

contribute to the computation of the relationships of the organisms. Thus all the organisms were sensitive to all the antibiotics at the concentration tested; none fermented dulcitol, produced lecithinase, decarboxylated any of the amino acids, produced acetoin or acetic acid in detectable amounts by the methods used to analyse the products of glucose fermentation, hydrolysed coagulated serum, utilized paraffins or grew with inorganic nitrogen as sole nitrogen source.

Table 2. *Growth ability features*

Ability to grow:	
Using inorganic nitrogen	
On peptone agar	
On nutrient agar	
On casitone yeast-extract agar	
On brain heart yeast extract agar	
In defined medium (Pine & Watson, 1959)	
At pH values from 5.5 to 8.5	
At 24° and 42°	
Aerobically and/or	} Technique of Howell & Pine, 1956
Anaerobically	
In various CO ₂ concentrations	
In presence of:	
Sodium taurocholate (0.1 and 0.25 %, w/v)	
Potassium tellurite (0.02 and 0.03 %, w/v)	
Sodium chloride (2.0 and 3.0 %, w/v)	
Crystal violet (final concentrations 10 ⁻⁵ , 10 ⁻⁶ , 10 ⁻⁷)	
Antibiotics (1 mg./100 ml. in ditch plates):	
streptomycin, chloramphenicol, aureomycin,	
terramycin, penicillin	

Table 3. *Biochemical features*

Acid production from carbohydrates
Final pH value and end products of fermentation of glucose
Utilization of organic acids
Utilization of tyrosine, xanthine, paraffins
Production of catalase
Production of cytochrome component (Deibel & Evans, 1960)
H ₂ S production
Nitrate reduction
Starch hydrolysis
Reduction of methylene blue
Proteolysis of gelatin, serum, casein
Indole production
Decarboxylation of amino acids (Møller, 1955; Stewart, 1963)
Urease and lecithinase production
Phosphatase production (Bray & King, 1943; Howell & Fitzgerald, 1953)
Haemolysis of horse blood

Computer analysis. The analysis was carried out on a Mercury computer according to a programme kindly made available by the University of London Computer Unit. This programme was similar to that of Sneath (1957) except that negative matches were counted as similarities for the reasons presented by Hill, Turri, Gilardi & Silvestri (1961). Quantitative data were scored by the additive method of Sneath (1962) since this appears to be the logical sequel to accepting negative matches. Quantitative data and multiple alternatives for a feature were limited to a maximum of four. Two complete analyses were made, one covering the 49 isolates capable of

good aerobic growth and the other covering the 65 isolates capable of good anaerobic growth: 43 strains of the isolates grew sufficiently well in tests done under both sets of growth conditions to be included in both analyses.

RESULTS

1. Forty-seven of the 71 isolates studied fell into two phenons A and B, each was composed of very similar organisms or of organisms more like the organisms in these two phenons than any others in the series.

Table 4. *Mean percentage similarity values within and between phenons*

No. of strains	Phenon											
	A		B		C		D		E1		E2	
	21	23	2	11	4	4	7	6	4	4	3	1
Phenon A	85											
		84										
B	74		94									
		75		83								
C	61		59		87							
		59		64		86						
D	58		57		70		84					
		59		63		66		78				
E1	69		72		72		64		83			
		70		71		71		65		82		
E2	59		66		67		60		75		83	
		58		71		67		65		69		100

Roman figures indicate similarity aerobically, while italics indicate similarity anaerobically.

Phenon A contained 24 isolates each of which showed a mean similarity value (MSV) to all the other isolates in the phenon in the range 83–88 % aerobic (based on analysis using aerobic results) and 82–87 % anaerobic (based on anaerobic tests). All these organisms grew to some extent under aerobic and anaerobic conditions but one grew too weakly aerobically and three anaerobically to be put through the full series of tests under these conditions. Phenon B contained 11 isolates with a MSV between 82 and 84 % (anaerobic tests). Only two grew aerobically to any degree with a high similarity to each other of nearly 94 %.

A further 12 isolates were more closely related to phenons A and B than to any others established, but the similarity was less close. Phenons A and B showed a mean interphenon similarity value (MIS) of all the organisms in one phenon to all the organisms in the other of 75 % (anaerobic tests).

2. Four isolates formed phenon C and each showed a MSV between 83–89 % (aerobic) and 83–87 % (anaerobic). All grew well both aerobically and anaerobically.

3. Seven isolates formed phenon D and each showed a MSV between 80–88 % (aerobic) and 75–80 % (anaerobic). One isolate grew poorly anaerobically and was not included in this series; the whole group was preferentially aerobic. Two other aerobic strains were less closely related to this phenon but were more like these than other organisms in the series.

4. Two small groups of 4 and 3 isolates formed phenons E1 and E2, respectively; these phenons showed a MIS of 75 % (aerobic). The organisms in E1 showed a MSV

of 81–85 % (aerobic) and of 82 % (anaerobic); those in E2 showed a MSV of 82–84 % (aerobic) but only one isolate was suitable for anaerobic testing. Two other isolates were more like isolates in E1 than the others investigated.

5. Two isolates were completely unplaced and showed low similarity values to all groups; one of these was unidentified, the other was an oral lactobacillus included for comparison.

DISCUSSION

Although similarity values have no absolute correlation with taxonomic rank the phenons suggested by overall similarity values may be compared with previous results arrived at by more classical methods of taxonomy. Phenons A and B and the organisms similar to, but not included in, these phenons appear to correspond to the genus *Actinomyces*. Within this genus, phenon A conforms to the description of *A. naeslundii* as expanded by Howell, Murphy, Paul & Stephan (1959) except that some strains grew more easily under aerobic conditions and, especially under these conditions, produced catalase. Three strains kindly supplied by Dr A. Howell did in fact fall into this phenon.

Phenon B conforms in general to the description of *Actinomyces bovis* and contains several isolates described by Pine, Howell & Watson (1960). Two strains of *A. odontolyticus* isolated by Batty (1958) also fell in this phenon; this supports the close relationship of *A. odontolyticus* to *A. bovis* suggested by Batty on the grounds of similarity of life cycle. It may be an indication that there is insufficient justification for the establishment of *A. odontolyticus* as a separate species, but it should be borne in mind that only two strains of these organisms were included and that they were the only isolates in the phenon which grew well aerobically.

The organisms similar to, but not included in, phenons A and B include all the strains which showed the typical description of *Actinomyces israelii* including the cell-wall composition described by Cummins & Harris (1958), the strain isolated by Buchanan & Pine (1962) and named *A. propionicus* and various isolates not very different from the organisms in phenons A and B.

The other phenons derived from the analysis of similarities each contained very few isolates. Phenon C corresponds to the whip-handle type of Leptotrichia which was named *Leptotrichia dentium* by Davis & Baird-Parker (1959) and *Bacterionema matruchotii* by Gilmour, Howell & Bibby (1961). The present work does not help directly to settle this controversy since the other main oral Leptotrichia (*L. buccalis*) was not included in the survey. The phenon is however more closely related to phenons D, E1 and E2 than it is to the genus *Actinomyces*, while all four isolates in the phenon showed very low similarity values to the lactobacillus included for comparison. This would suggest that the whip-handle type should be placed more nearly with the nocardias or corynebacteria than with the actinomyces and that it bears little similarity to *L. buccalis* if the contention of Richardson & Schmidt (1959) that *L. buccalis* should be placed near to the genus *Lactobacillus* is correct.

Phenon D corresponds to, and includes specimens of, the isolates named by Davis & Freer (1960) as *Nocardia salivae* and which differ from typical nocardias in cell-wall composition. No nocardias from other sources were included in the analysis so that the position of *N. salivae* in this genus is neither confirmed or disputed. Phenons E1 and E2 are as closely related as are phenons A and B and from the general properties

of the organisms included in them may well be members of the genus *Corynebacterium*; the number of isolates studied was insufficient to allow further comment particularly in view of the complexity of overall similarity analysis of this group shown by Moore & Davis (1963).

In general the phenons established are too small to allow conclusions to be drawn from a detailed study of the properties of the individual isolates in each group. It is of interest that the facultative isolates fell into the same groups whether their features were determined aerobically or anaerobically, and that certain organisms which were preferentially aerobic or anaerobic appeared in the same group due to their similarity to facultative strains. The experimental evidence also supported the view of Roth & Thurn (1962) that catalase production is adaptive rather than constitutive and indicated that production of this enzyme should not, on its own, be sufficient ground for removing an organism from the genus *Actinomyces*; certainly some micro-organisms placed in phenon A are capable of producing catalase.

REFERENCES

- BATTY, ISABEL (1958). *Actinomyces odontolyticus*, a new species of actinomycete regularly isolated from deep carious dentine. *J. Path. Bact.* **75**, 455.
- BRAY, J. & KING, E. J. (1948). The phosphatase reaction as an aid to the identification of micro-organisms using phenolphthalein phosphate as substrate. *J. Path. Bact.* **55**, 315.
- BUCHANAN, B. B. & PINE, L. (1962). Characterization of a propionic acid producing actinomycete, *Actinomyces propionicus*, sp. nov. *J. gen. Microbiol.* **28**, 305.
- BUCHANAN, R. E. (1925). *General Systematic Bacteriology*. Baltimore: Williams and Wilkins Co.
- CUMMINS, C. S. & HARRIS, H. (1956). The chemical composition of the cell-wall 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. & BAIRD-PARKER, A. C. (1959). Bacterial elements of materia alba. *Brit. dent. J.* **106**, 142.
- DAVIS, G. H. G. & FREER, J. H. (1960). Studies upon an oral aerobic actinomycete. *J. gen. Microbiol.* **23**, 163.
- DEIBEL, R. H. & EVANS, J. B. (1960). Modified benzidine test for the detection of cytochrome-containing respiratory systems in micro-organisms. *J. Bact.* **79**, 356.
- GILMOUR, MARION N., HOWELL, A. & BIBBY, B. G. (1961). The classification of organisms termed *Leptotrichia* (*Leptothrix*) *buccalis*. *Bact. Rev.* **25**, 131.
- HILL, L. R., TURRI, M., GILARDI, E. & SILVESTRI, L. (1961). Quantitative methods in the systematics of Actinomycetales. II. *G. Microbiol.* **9**, 56.
- HOWELL, A. & FITZGERALD, R. J. (1953). The production of acid phosphatases by certain species of Actinomyces. *J. Bact.* **66**, 437.
- HOWELL, A., MURPHY, W. C., PAUL, FRANCES & STEPHAN, R. M. (1959). Oral strains of Actinomyces. *J. Bact.* **78**, 82.
- HOWELL, A. & PINE, L. (1956). Studies on the growth of species of Actinomyces. 1. Cultivation in a synthetic medium with starch. *J. Bact.* **71**, 47.
- MELVILLE, T. H. (1964). *A study of the overall similarities or differences in certain oral actinomycetes*. University of Liverpool; Ph.D. Thesis.
- MØLLER, V. (1955). Simplified tests for some amino acid decarboxylases and for the arginine dihydrolase system. *Acta path. microbiol. scand.* **36**, 158.
- MOORE, K. & DAVIS, G. H. G. (1963). Taxonomy and incidence of oral Corynebacteria. *Brit. dent. J.* **114**, 254.
- PINE, L., HOWELL, A. & WATSON, SARAH J. (1960). Studies of the morphological, physiological and biochemical characters of *Actinomyces bovis*. *J. gen. Microbiol.* **23**, 403.

- PINE, L. & WATSON, SARAH J. (1959). Evaluation of an isolation and maintenance medium for Actinomyces and related organisms. *J. Lab. clin. Med.* **54**, 107.
- RICHARDSON, R. L. & SCHMIDT, JEAN (1959). An oral filamentous organism: cultural characteristics and microbial relationships affecting growth. *J. dent. Res.* **38**, 1016.
- ROTH, GENEVIEVE D. & THURN, A. N. (1962). Continued study of oral Nocardia. *J. dent. Res.* **41**, 1279.
- SNEATH, P. H. A. (1957). The application of computers to taxonomy. *J. gen. Microbiol.* **17**, 201.
- SNEATH, P. H. A. (1962). The construction of taxonomic groups. In *Microbial Classification, Symp. Soc. gen. Microbiol.* **12**, 289.
- STEWART, D. J. (1963). A rapid and unequivocal method for detecting glutamic acid decarboxylase in primary plate cultures of Enterobacteriaceae. *J. appl. Bact.* **26**, 75.