Numerical Taxonomy and Laboratory Identification of *Actinomyces* and *Arachnia* and some Related Bacteria

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SUMMARY

A numerical taxonomic study was made on 49 facultative anaerobic Gram-positive filamentous and/or diphtheroidal organisms isolated from dental plaques, carious dentin and faeces, together with 63 reference strains belonging to the genera *Actinomyces*, *Arachnia*, *Bifidobacterium*, *Actinobacterium*, *Propionibacterium*, *Eubacterium* and *Lactobacillus*. They were examined for 90 unit characters covering a wide range of tests and properties. The data were subjected to computer analysis in which the simple matching coefficient (S_{sm}) and the similarity index (S_j) were calculated, and the results of single linkage techniques and an unweighted average linkage cluster analysis technique were compared.

The strains fell into six major groups (phena). The *Actinomyces* strains were recovered in two phena; the first contained *Actinomyces israelii* and the other facultative anaerobic *Actinomyces*, including subclusters equal to taxospecies of *A. odontolyticus* and *A. viscosus/A. naeslundii*, while the other phenon corresponded to the genera *Arachnia*, *Actinobacterium*, *Bifidobacterium* and *Propionibacterium*. The groups of *Arachnia* and *Actinobacterium* each contained one species, representing taxospecies of *Arachnia propionica* and *Actinobacterium meyerii*. Taxonomic criteria, both constant and discriminative, were selected to form a diagnostic table useful for laboratory identification of this group of organisms. Immunofluorescence supported the numerical data.

INTRODUCTION

The classification of Gram-positive diphtheroidal and/or filamentous organisms belonging to the family Actinomycetaceae and related genera is beset with many difficulties. The lines of demarcation between the actinomycete genera, and between them and related genera such as *Propionibacterium* and *Actinobacterium*, are not clear. Current taxonomic ideas concerning the family Actinomycetaceae and related organisms, and the physiology and structure of these organisms in relation to their classification and phylogenetic relationships were recently reviewed by Pine (1970) and by Cross & Goodfellow (1973).

Few analyses have used numerical taxonomy for the classification of actinomycetes (Sneath, 1970). Melville (1965), in a numerical taxonomic study on oral actinomycetes, separated *Actinomyces naeslundii*, *Bacterionema matruchotii* and *Nocardia salivae* (*Rothia dentocariosa*) but did not recover well-defined groups of *A. israelii*, *A. odontolyticus* and *A. bovis*. Holmberg & Hallander (1973) recognized well-defined groups of *Rothia*, *Bacterionema*, *Nocardia* and *Corynebacterium* equivalent to generic taxonomic status. A heterogeneous generic cluster of *Actinomyces* strains included a distinct taxospecies of *A. naeslundii*, *A. viscosus*, and a less well-defined group of *A. israelii* and *Ar. propionica*.

Lack of reliable differentiating tests based on good classifications has impeded the identification of the actinomycetes. Workers in clinical microbiology laboratories have been

forced to depend on morphological criteria that tend to be highly variable. The ability to identify two anaerobic actinomycetes, *A. israelii* (Wolff & Israel, 1891) and *Ar. propionica* (Pine & Georg, 1969), that overtly produce disease in man (Georg, 1970; Brock *et al.* 1973) is important when evaluating their distribution and potential roles in healthy and diseased body sites. These organisms are morphologically similar to other actinomycetes of unproven pathogenicity in man (Georg, 1970; Bowden & Hardie, 1973). During the past 15 years several investigators have described a number of physiological tests for differentiating the clinically important species of Actinomyces and related bacteria (Georg, Roberstad & Brinkman, 1964; Gerencser & Slack, 1969; Slack, Landfried & Gerencser, 1969; Georg, 1970). More recently, direct immunofluorescence (Lambert, Brown & Georg, 1967; Blank & Georg, 1968; Brock & Georg, 1969*b*; Slack *et al.* 1969; Holmberg & Forsum, 1973), gas chromatographic analysis of metabolic end-products in glucose fermentation (Li & Georg, 1968) and paper chromatography of whole cell hydrolysates (Boone & Pine, 1968) have been introduced as an aid to identification. These new tests must now be included and evaluated in numerical taxonomic studies.

The present study was concerned with an assessment of the relationships, the construction of natural groups, and the differentiation and identification of Actinomyces and Arachnia species from related bacteria by using the method of numerical taxonomy (*Methods for Numerical Taxonomy*, 1970; Sneath, 1972; Sneath & Sokal, 1973). The study combines a numerical analysis of the overall phenetic resemblance of fresh isolates and reference strains with an immunofluorescence analysis.

METHODS

Organisms. A total of 42 fresh isolates of facultative anaerobic Gram-positive non-sporeforming filamentous and/or diphtheroidal bacteria were selected from dental plaques (Holmberg, 1975) and faeces. Ten organisms typed as Actinomyces strains, seven isolates of anaerobic Gram-positive, non-sporeforming rods isolated from dentin caries and a number of reference strains were included in the study (Table 1). The type strain of *Clostridium perfringens*, ATCC13124, was included to orientate it in relation to sporeforming anaerobic Gram-positive rods. The names of strains from culture collections were used according to their designations. To afford a check on the test reproducibility, one *Actinomyces naeslundii* strain (designated ATCC12104 and NCTC10301), one *A. odontolyticus* strain (designated ATCC17982 and NCTC9931), and one *Actinomyces israelii* strain (designated Holm 1452/45-56 and VPI3258), were replicated. All bacteria were maintained in CO₂-gassed tubes containing pre-reduced anaerobically sterilized (PRAS) peptone-yeast extract (PY) broth (*Anaerobe Laboratory Manual*, 1973), consisting of: Bacto-peptone (Difco), 10 g; yeast extract (Difco), 10 g; resazurin solution, 0.4 ml; salt solution, 40 ml; purified agar, 1.6 g; distilled water, 1 l; cysteine-HCl.H₂O, 0.05 g; pH 7.0 ± 0.2. They were also stored lyophilized. The cultures were checked for purity at intervals throughout the study.

Methods of testing. The organisms were grown in PRAS PY broth for 5 days. The cultures were centrifuged and the organisms washed and suspended in pre-reduced salt solution, pH 7.2 (*Anaerobe Laboratory Manual*, 1973), to give an extinction of approximately 0.2 at 540 nm, on a Coleman Junior spectrophotometer (Coleman Instrument Inc., Illinois, U.S.A.). The environmental anaerobic conditions were maintained during these procedures by the use of the Virginia Polytechnical Institute (VPI) anaerobic culture system (Bellco Glass Co., Vineland, New York, U.S.A.) (Moore, 1966). The bacterial suspension was then drawn into a CO₂-gassed injection syringe, and approximately 0.2 ml of the suspension inoculated into PRAS test media in CO₂-gassed tubes sealed with butyl rubber

Table 1. *List of organisms*

Organisms	Source*/no.	Organisms	Source*/no.
<i>Actinomyces viscosus</i>	ATCC15987	<i>P. acnes</i>	NCTC737
<i>A. viscosus</i>	ATCC19246	<i>P. acnes</i>	CDC554
<i>A. naeslundii</i>	ATCC12104	<i>P. acnes</i>	CDC605
<i>A. naeslundii</i>	NCTC10301	<i>P. avidum</i>	ATCC25577
<i>A. odontolyticus</i>	ATCC17982	<i>P. avidum</i>	CDC9064
<i>A. odontolyticus</i>	WVU482	<i>P. granulosum</i>	ATCC25564
<i>A. odontolyticus</i>	NCTC9931	<i>Lactobacillus brevis</i>	ATCC14869
<i>A. odontolyticus</i>	NCTC9935	<i>L. fermentum</i>	ATCC14931
<i>A. israelii</i>	ATCC12103	<i>L. casei</i> var. <i>rhamnosus</i>	ATCC7469
<i>A. israelii</i>	NCTC8047	<i>L. catenaforme</i>	ATCC25536
<i>A. israelii</i>	WVU307	<i>Eubacterium limosum</i>	ATCC8456
<i>A. israelii</i>	VPI3258	<i>E. lentum</i>	ATCC25559
<i>A. israelii</i>	PHLS A9	<i>E. ventriosum</i>	ATCC27560
<i>A. israelii</i>	PHLS A18	<i>Clostridium perfringens</i>	ATCC13124
<i>A. israelii</i>	PHLS A26	<i>Propionibacterium</i> strain received as	
<i>A. bovis</i>	ATCC13863	<i>Corynebacterium acnes</i> group 2	D34 (Voss)
<i>Arachnia propionica</i>	ATCC14157	<i>Actinomyces</i> isolates	104/45-46 (Holm)
<i>Ar. propionica</i>	WVU346		169/45-46 (Holm)
<i>Ar. propionica</i>	PHLS A28		175/45-46 (Holm)
<i>Actinobacterium meyerii</i>	VPI0003		1061/40-41 (Holm)
<i>Act. meyerii</i>	VPI8611		1452/40-41 (Holm)
<i>Act. meyerii</i>	VPI8617		1654/40-41 (Holm)
<i>Act. meyerii</i>	VPI8620B		1919/40-41 (Holm)
<i>Act. meyerii</i>	VPI8622		2129/40-41 (Holm)
<i>Act. abscessum</i>	Pasteur 4567		A555/74 (Bodenhoff)
<i>Bifidobacterium breve</i>	ATCC15700		A752/74 (Bodenhoff)
<i>B. liberorum</i>	ATCC15702	Anaerobic 'diphtheroid' isolates from dentin caries	Be8, Be15, Be18, Be25, Be26, Be31, Be47,
<i>B. adolescentis</i> var. a	ATCC15703	26 own anaerobic 'diphtheroides' from dental plaques	56 to 81
<i>B. adolescentis</i> var. b	ATCC15704	16 own anaerobic 'diphtheroides' from faeces	82 to 97
<i>B. adolescentis</i> var. c	ATCC15705		
<i>B. adolescentis</i> var. d	ATCC15706		
<i>B. longum</i>	ATCC15707		
<i>B. eriksonii</i>	ATCC15423		
<i>B. bifidum</i>	ATCC15696		
<i>B. infantis</i>	ATCC15697		
<i>B. parvulorum</i>	ATCC15698		
<i>B. dentium</i>	ATCC27534		
<i>Propionibacterium acnes</i>	ATCC11828		

* ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; NCTC, National Collection of Type Cultures, Colindale, London; CDC, Center for Disease Control, Atlanta, Georgia, U.S.A.; WVU, West Virginia University, Medical School, Morgantown, West Virginia, U.S.A.; PHLS, Public Health Laboratory Service, London; VPI, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, U.S.A.; Voss, Dr J. G. Voss, The Procter & Gamble Co., Miami Valley Laboratories, Cincinnati, Ohio, U.S.A.; Pasteur, Institut Pasteur, Paris, France; Holm, Dr P. Holm, State Serum Institute, Copenhagen, Denmark; Bodenhoff, Dr J. Bodenhoff, State Serum Institute, Copenhagen, Denmark; Be, Dr G. Bowden, London Hospital Medical College, London.

membranes and screw caps (Attebery & Finegold, 1969; Macy, Snellen & Hungate, 1972), and on to one aerated peptone-yeast extract-glucose (PYG; *Anaerobe Laboratory Manual*, 1973) agar plate.

Colony morphology was examined on PRAS PYG agar slants under $\times 10$ stereomagnification. Size, shape and appearance of bacteria was determined by dark-field microscopy by using wet preparations from the PRAS PYG agar slants. Gram reaction was examined on smears stained by Hucker's modification (Cowan & Steel, 1970). Anaerobic growth at 45 °C in PRAS PYG broth was noted after 5 days. Spores were detected in Gram-

stained preparations, and motility observed in drops of PYG broth on slides by dark-field microscopy. Catalase activity was tested on the anaerobic PYG agar slant, after exposure to air for at least 30 min, by the addition of 3 % (w/v) H_2O_2 and observation of the evolution of gas bubbles.

Nitrate reduction, indole production, aesculin hydrolysis, liquefaction of gelatin, starch hydrolysis, H_2S production and lecithinase production were demonstrated after incubation for 5 days at 37 °C in media and reagents devised by Holdeman and Moore (*Anaerobe Laboratory Manual*, 1973). Reduction of nitrate to nitrite was detected in oxygen-free indole-nitrite medium (BBL) without cysteine-HCl and resazurin, by adding 0.5 ml of 1.0 % (w/v) sulphanilic acid in 5 M-acetic acid, followed by 0.5 ml of 0.6 % (w/v) dimethyl- α -naphthyl-amine in 5 M-acetic acid. The development of red colour indicated a positive result. False negatives were avoided by adding approximately 1 mg zinc dust/ml culture. Indole production in PY medium was detected by Kovac's reagent. The development of a pink to red colour in the reagent was recorded positive. Aesculin hydrolysis was studied in tubes containing PY broth with 0.5 % (w/v) aesculin. Hydrolysis was demonstrated by adding drops of an aqueous 1.5 % (w/v) ferric citrate solution. Liquefaction of gelatin, demonstrated by the failure to solidify in the refrigerator after incubation as compared with controls of uninoculated tubes of gelatin, was recorded as positive. Hydrolysis of starch was tested by means of PY starch medium and Lugol's iodine solution. The test for H_2S production was made in a medium devised by Holdeman and Moore (*Anaerobe Laboratory Manual*, 1973). Development of black colour in the medium after incubation was recorded as H_2S production. Lecithinase production was demonstrated in egg-yolk medium (Difco). A positive lecithinase reaction was indicated by an opaque zone in the medium around the colonies.

Ammonia from arginine (1.0 %, w/v, added to PY medium) was detected by Nessler's solution. Voges-Proskauer and the methyl-red tests were done in PYG medium without resazurin according to Cowan & Steel (1970). All of the test media were semi-solid (0.1 %, w/v, agar) and poured into screwcapped tubes, except for the egg-yolk and starch media and the Rogosa-SL medium (Difco) which were solidified with 2 % (w/v) agar and prepared in plates. All plates were stored anaerobically until inoculated, after which they were immediately incubated in anaerobic GasPac jars.

Acid production from carbohydrates was tested in a basal semi-solid PRAS PY medium. Carbohydrates were added to this basal medium at concentrations of 1 % (w/v), except for arabinose, adonitol, glycogen, ribose and trehalose which were added at 0.5 % (w/v). Media were poured into CO_2 -gassed screwcapped tubes. Syringe-inoculated tubes were incubated for 5 days at 37 °C and acid production was detected by determining the pH directly in the tubes with a pH meter (Radiometer AS, Copenhagen, Denmark). pH values below 5.7 were recorded as positive. Weak acid production (pH 5.8 to 5.9) was considered negative since the pH of uninoculated PRAS PYG media containing arabinose, ribose and xylose was found to be about 6.0 to 6.2 after standing 1 to 2 days in the CO_2 atmosphere.

Analyses of bacterial metabolic end-products from glucose fermentation were studied in a 5-day-old PYG broth culture containing 5 g glucose/l. Ether extraction and methylation of acidic metabolic components from the culture were performed as described by Holdeman and Moore (*Anaerobe Laboratory Manual*, 1973). The results obtained from gas-liquid chromatography on two different instruments were compared. A Varian Aerograph model 940 (Varian Associates, California, U.S.A.), equipped with a hydrogen-flame ionization detector, employed a $5\text{ ft} \times \frac{1}{8}$ in outer diameter stainless-steel column containing 10 % (w/w) FFAP (Applied Science Laboratory, Incorporated, Pennsylvania, U.S.A.) on

100/120 Varaport. The operation parameters of the instrument were as follows: injection-
port temperature 200 °C, detector temperature 200 °C, column-oven temperature 115 °C.
The carrier gas was 10 ml nitrogen/min, and a 3.0 µl sample containing the test substance
was injected into the instrument. The other instrument was a Varian Aerograph model 920
fitted with a thermal-conductivity detector. A 5 ft × ¼ in stainless-steel column packed with
5 % (w/w) FFAP on Cromosorb G 70/80 AM-DMCS was used. The operating temperatures
for the injector, detector and column were 200, 160 and 120 °C, respectively. The carrier
gas was 50 ml helium/min, and a 15 µl sample was injected. An uninoculated PYG-medium
blank was tested to detect the presence of contaminating acids. If acids were present in
significant quantities the medium was discarded, but if there were only trace amounts the
blank was subtracted from the culture results. Identification was achieved by comparing the
retention times of fermentation products with those of standard mixtures. Formic acid was
not detected by the gas-chromatograph equipped with a flame-ionization detector.

Computer analysis. The data were analysed by using a numerical taxonomy program
written for an IBM 1130 computer. Characters existing in one of two mutually exclusive
states were coded in binary form (0 and 1) for computation. Quantitative multi-state
characters were converted into two-state characters using the additive coding method
(Sneath, 1957). Thus, the results were coded in 90 character states. Nineteen of these gave
identical results and were deleted from the computation. All strains were Gram-positive and
did not form square-ended cells. No colonies exhibited an eroded or fimbriated margin. All
strains grew anaerobically in PRAS PYG medium at 37 °C. No strain produced isobutyric,
valeric, isocaproic, heptanoic, pyruvic, oxalacetic, oxalic, methyl malonic or fumaric acid
from glucose in PRAS PYG broth. Thus, the final $n \times t$ matrix contained 71 character states
and 112 strains or operational taxonomic units (o.t.u.s).

The phenetic resemblance for each pair of o.t.u.s was calculated in two ways: by the
similarity coefficient (S_s) (Sneath, 1957), in which negative similarities are excluded; and by
the simple matching index (S_{sm}) (Sokal & Michener, 1958), in which negative similarities
are included. The percentage difference between strains was estimated by calculating the
vigour difference, D_v , and the pattern difference, D_p , as devised by Sneath (1968). The
confidence limits of the resemblance coefficients were estimated as the sum of the effects on
the similarity coefficient of the experimental error (Sneath & Johnson, 1972) and the
sampling error (Goodall, 1967). The single-linkage cluster analysis (SLCA) (Sokal &
Sneath, 1963) and the unweighted pair group method using arithmetic averages (UPGMA)
(Sokal & Michener, 1958) were employed for clustering. The co-phenetic correlation tech-
nique (Sokal & Rohlf, 1962) was used to calculate the agreement between the similarity
and dissimilarity matrices and the dendrograms derived by the SLCA and UPGMA
clusterings. The homogeneity of the defined clusters (phena) and the inter-relationships
between them were determined by calculating the intra- and inter-group similarity values.
The differences between the phena due to variations in total metabolic activity, as
measured by the number of positive reactions, were analysed by calculating the mean,
range and standard deviation of vigour values and by estimating the spread between the
phena. The effect of vigour on the phenetic relationships between strains was estimated
by clustering on the D_p coefficients matrix by SLCA and UPGMA and comparing the
relationships of strains with those obtained by clustering on the similarity coefficients.
The frequency of occurrence of the characters in the 'natural' polythetic groups (phena)
was tabulated. A 'calculated median organism' (c.m.o.) for each phenon equivalent to
species rank was calculated according to Liston, Weibe & Colwell (1963), and the dimen-
sions of the taxospecies were calculated as a measure of the phenetic variation of the taxa.

The range of variation within a taxospecies was obtained by assuming that the similarity values of the strains to the c.m.o. showed a normal distribution curve around the mean similarity value to the c.m.o. It was further expected that 95 % of the members of a phenon would be allocated within the mean similarity value $\pm$ twice the standard deviation (Gyllenberg, 1965). The lowest permissible similarity index for a strain to be included in a particular species was established.

Distinguishing and determining features, suitable for providing an identification scheme, were selected by comparing the total array of characters for each defined cluster of strains. A differential weighting of characters for the construction of a diagnostic table was performed on the basis of their constancy within the taxa, with frequencies in the taxa of less than 10 % or above 90 %.

Immunofluorescence studies. Immunofluorescent staining of pure cultures was used to check the computed strain clusters. Monospecific and fractionated fluorescein-isothiocyanate (FITC)-labelled rabbit IgG or F (ab')₂-fragments of the IgG antibodies were prepared against *A. viscosus* serotype 1 (ATCC15987), *A. viscosus* serotype 2 (ATCC19246), *A. naeslundii* (ATCC12104), *A. odontolyticus* serotype 1 (ATCC17982), *A. odontolyticus* serotype 2 (WVU482), *A. israelii* serotype 1 (ATCC12103), *A. israelii* serotype 2 (WVU307), *Ar. propionica* serotype 1 (ATCC14157), *Ar. propionica* serotype 2 (WVU346), *Actinobacterium meyerii* (VPI8617), *Propionibacterium acnes* serotype 1 (NCTC737) and *P. acnes* serotype 2 (ATCC11828). All conjugates exhibited the physicochemical and performance characteristics outlined by Holmberg & Forsum (1973). Smears were stained and the results recorded as detailed in the same report. All smears were read under a Zeiss fluorescence microscope equipped with an Osram HB200 mercury lamp, an oil-immersion dark-field condenser, and a combination of a 4 mm BG 12 primary filter and a Zeiss 50 secondary filter.

RESULTS

Clusterings of the strains

The results of the computation are presented in a dendrogram (Fig. 1), derived by SLCA of the sorted matrix calculated by using the S_{SM} matching coefficient. The co-phenetic correlation was 0.89. Ninety-four of 112 strains (84 %) were assigned to one of six main clusters (phena) recognized at the range of 85 to 88 % similarity level (Table 2). The phena were given the names of the numerically dominant reference strains found within the clusters. Thus, three of the major phena could be equated with the genera *Bifidobacterium*, *Arachnia* and *Propionibacterium*. In one of the remaining phena, reference strains of the facultative anaerobic Actinomycetes species, *A. viscosus*, *A. naeslundii* and *A. odontolyticus*, were grouped. The other two phena contained named strains of *A. israelii* and *Act. meyerii*, respectively.

Phenon 1: the Bifidobacterium cluster. This cluster contained twelve new isolates from faeces, dental plaques and dentin caries, and all the reference strains of *Bifidobacterium* species except *B. bifidum*. Three sub-clusters are discernible. Within one of the sub-clusters, the reference strains of *B. longum* formed a distinct subgroup with four strains (88, 94, 95 and 96) isolated from faeces. Within one of the other sub-clusters, the reference strains of *B. adolescentis* var. b, *B. dentium* and *B. eriksonii* formed a subgroup together with two new isolates, one from dental plaques (80) and one from dentin caries (Be18). Two strains (57 and 58) isolated from dental plaques, and the reference strains *B. adolescentis* var. d, *B. parvulorum* and *B. infantis*, were joined in a subgroup at 91 % similarity. The remaining reference strains and isolates did not form subgroups but were linked to the sub-clusters at the 89 % similarity level.

Fig. 1. Simplified dendrogram of relationships between the main phena formed by SLCA on *S_{SM}*. The top of a filled column represents the lowest similarity at which any branch arises among the strains in the phenon.

Phenon 2: the Actinomyces israelii cluster. Six new isolates from dental plaques and 18 reference strains joined above the 91 % similarity level to form a tight cluster. A further strain, Be26, joined this phenon at 89 % similarity.

Phenon 3: the Actinobacterium meyerii cluster. Five reference strains, designated *Act. meyerii*, formed a tight cluster at the 94 % similarity level.

Phenon 4: the facultative anaerobic Actinomyces cluster. This cluster consisted of 11 strains. Two distinct and contrasting sub-clusters, 4A and 4B, were defined at the 92.5 %

Table 2. *Strains of the major groups (phena)*

Phenon	No. of strains	Strain nos.	Designation of phenon
1	23	ATCC15697, ATCC15700, ATCC15702, ATCC15703, ATCC15704, ATCC15705, ATCC15706, ATCC15423, ATCC15707, ATCC15698, ATCC27534, 57, 58, 65, 80, 88, 89, 90, 92, 94, 95, 96, Be18	<i>Bifidobacterium</i> group
2	24	ATCC12103, WVU307, A555/73 (Bodenhoff), A752/73 (Bodenhoff), 104/45-46 (Holm), 169/73 (Holm), 175/45-46 (Holm), 1061/40-41 (Holm), 1452/40-41 (Holm), 1654/40-41 (Holm), 1919/40-41 (Holm), 2129/40-41 (Holm), NCTC8047, VPI3258, PHLS A18, PHLS A9, PHLS A26, 66, 69, 71, 75, 76, 78, Be15	<i>Actinomyces israelii</i>
3	5	VPI0003, VPI8611, VPI8617, VPI8620B, VPI8622	<i>Actinobacterium meyerii</i>
4A	5	ATCC15987, ATCC19246, ATCC12104, NCTC10301, 68	<i>Actinomyces viscosus</i> / <i>A. naeslundii</i>
4B	6	ATCC17982, NCTC9931, NCTC9935, WVU482, Be31, 60	<i>Actinomyces odontolyticus</i>
5	8	ATCC14157, WVU346, PHLS A28, 62, 63, 72, 81, Be8	<i>Arachnia propionica</i>
6	22	NCTC737, ATCC11828, D34 (Voss), ATCC25577, ATCC35564, CDC554, CDC605, CDC9064, 56, 59, 61, 64, 67, 74, 77, 82, 83, 84, 86, 87, 97, Be47	<i>Propionibacterium</i> group
6A	14	NCTC737, ATCC11828, CDC554, CDC605, 56, 59, 61, 64, 67, 74, 77, 82, 83, 97	<i>Propionibacterium acnes</i>

For abbreviations, see Table 1.

similarity level or above. Sub-cluster 4A comprises the four reference strains of *Actinomyces viscosus* and *A. naeslundii*, and one isolate from dental plaques. Included in sub-cluster 4B were the four reference strains of *A. odontolyticus*, one isolate from dentin caries and one from dental plaques. Isolate 73, and the reference strain *A. bovis* ATCC13863, fell just outside phenon 4.

Phenon 5: the *Arachnia* cluster. This cluster represented *Arachnia propionica*. It contained the three reference strains received as *Ar. propionica*, four isolates from dental plaques and one strain isolated from carious dentin, Be8. They formed a phenon at the 87 % similarity level.

Phenon 6: the *Propionibacterium* cluster. This cluster contained 22 strains. Isolates from faeces, dental plaques and carious dentin, and the reference strains received as *Propionibacterium* spp., were represented. A sub-cluster, 6A, included the reference strains of *P. acnes* and ten isolates which coalesced above the 91 % similarity level. A *Propionibacterium* strain received as *Corynebacterium acnes* group 2 (Voss D34), and the reference strain of *P. granulosum* (ATCC25564) were included at the periphery of this sub-cluster. The other strains, including named strains received as *P. avidum* (ATCC25577 and CDC9064) were linked at the 87 % similarity level.

The ungrouped strains. Five strains isolated from plaques and faeces, and one strain isolated from dentin caries, could not be assigned to any of the main clusters defined at the

Table 3. Mean similarity (S_{SM}) values within and between the phenons, and the vigour values

Phenons	Percentage similarity values, and S.D.										Vigour value		
	1	2	3	4	4A	4B	5	6	6A	Range	Mean	S.D.	
1	83.6 ±4.6									0.362-0.492	0.430	±0.051	
2	80.5 ±7.3	88.9 ±3.3								0.420-0.507	0.475	±0.023	
3	81.7 ±6.0	83.2 ±9.8	97.3 ±1.7							0.333-0.391	0.368	±0.021	
4	79.9 ±6.3	82.1 ±8.1	84.0 ±7.3	86.9 ±5.6						0.362-0.434	0.404	±0.030	
4A	80.8 ±6.4	84.5 ±7.8	84.1 ±10.1	—	92.1 ±3.6								
4B	80.9 ±5.8	84.1 ±7.6	86.8 ±7.1	—	—	91.1 ±3.6							
5	78.1 ±8.1	83.6 ±7.3	80.7 ±10.0	81.5 ±7.3	83.5 ±6.2	80.9 ±8.4	86.8 ±3.3			0.318-0.420	0.365	±0.032	
6	74.3 ±10.2	74.1 ±12.8	79.9 ±8.0	77.6 ±8.5	79.4 ±8.2	78.8 ±8.4	80.3 ±7.5	83.0 ±5.9		0.333-0.449	0.397	±0.037	
6A	74.8 ±11.2	75.3 ±14.4	82.0 ±9.5	78.2 ±10.1	80.5 ±10.4	80.1 ±10.2	81.8 ±7.5	—	88.1 ±3.7

Fig. 2. Dendrogram of relationships within and between phena formed by UPGMA clustering on S_{SM} .

86 % similarity level or above. The reference strains of the genera *Lactobacillus* and *Eubacterium*, and the reference strains of *B. bifidum* (ATCC15696), *Actinobacterium abscessum* (Pasteur 4567) and *Cl. perfringens* (ATCC13124), were also ungrouped.

The mean and the standard deviations of the intra- and inter-group similarity values of the defined phena are presented in Table 3. The range, mean and standard deviations of the vigour values are shown in the same Table. The differences in vigour between the major phena were small. The highest value is shown by *A. israelii* and the lowest by *Act. meyerii*, but the spread was not great. Clustering on D_P , the pattern difference coefficient, by SLCA gave results very similar to that obtained by clustering with SLCA on the similarity values (S_{SM}).

Comparison of data by means of different resemblance coefficients and cluster analysis techniques

The taxonomic relationship derived from the S_{SM} matrix by an unweighted average linkage (UPGMA) clustering was very similar (Fig. 2). The co-phenetic coefficient was 0.92 and the dendrogram revealed few additional points of interest. Ninety-three of the 112 strains were assigned together at the 78 % similarity level. The isolate be26 was now ungrouped. The six main phena defined by SLCA were recovered and delineated at the 78 to 83 % similarity level. They included the two sub-clusters 4A and 4B of the facultative anaerobic *Actinomyces* cluster (4). In contrast, the sub-cluster *P. acnes* (phenon 6A) was not recovered in its entirety. The strain D34 (Voss) was now included in this sub-cluster, whereas the isolate 64 was unplaced. The strain be47 was placed at the periphery of the sub-cluster 6A. In addition, the type strain of *B. eriksonii* (ATCC15423) and the isolate be18 were more distinctly separated from the subgroup containing the strains of *B. adolescentis* var. b and *B. dentium*.

A similarity matrix of coefficients was produced based on the similarity index, S_J , which only scores positive matches. The dendrograms produced by SLCA and UPGMA clustering from this similarity matrix revealed the six main phena. They were delineated as distinct clusters at the 78 % similarity level.

Characters of the defined phena

The major phena shared a series of salient and almost invariable properties (Table 4). All strains were non-motile, Gram-positive non-sporeforming irregular rods, often pleomorphic with club-shaped ends and dichotomous branching. All strains grew anaerobically in an oxygen-free CO₂ atmosphere in PRAS PYG medium, giving small (< 2 mm diam) rough or smooth convex colonies that were opaque, and commonly white or off-white to grey. All strains were fermentatively active and fermented glucose with the formation of acidic metabolic end-products.

All *Bifidobacterium* strains of phenon 1 formed irregular branched elements with club-shaped ends, and showed dichotomous or multiple branching. The colonies were smooth, and raised or convex, with entire or crenate margins. No strains grew under aerobic conditions. All strains hydrolysed starch but were unable to reduce nitrate. The fermentative pattern included the production of acid from galactose, lactose, raffinose and ribose. The end-products from glucose fermentation were acetic and lactic acids, approximately in the ratio 1.5 to 1.0. Some strains also produced succinic acid.

The *A. israelii* strains of phenon 2 showed markedly pleomorphic elements varying from cocco-bacillary to irregular rods in filamentous arrangement with rounded or club-shaped ends. The colonies were convex and rough with crenate margins, and mostly white or off-

Table 4. The characters of the major phena *A. viscosus*/*A. naeslundii*, *A. odontolyticus*, *A. israelii*, *Act. meyerii*, *Ar. propionica* and *P. acnes*, expressed as percentages of the strains exhibiting that character

Character	<i>A. viscosus</i> / <i>A. naeslundii</i>	<i>A. odontolyticus</i>	<i>A. israelii</i>	<i>Act. meyerii</i>	<i>A. propionica</i>	<i>P. acnes</i>
Cell morphology						
Coccus (spherical)	0	0	0	0	13	0
Cocco-bacillary	100	50	33	100	100	43
Regular rods	0	0	4	0	0	0
Irregular rods	100	100	100	100	100	100
Dichotomous	0	50	96	0	0	0
Multiple branching	20	0	21	0	0	0
Pleomorphic	100	100	96	100	75	0
Square-ended elements	0	0	0	0	0	0
Round-ended elements	100	17	96	100	100	100
Club-shaped ends	0	17	21	0	0	14
Tapered elements	100	0	33	100	88	100
Fusiform elements	0	0	0	0	0	0
Gram stain reaction negative	0	0	0	0	0	0
Gram stain reaction positive	100	100	100	100	100	100
Gram stain reaction variable	0	0	0	0	0	0
Single elements	100	100	82	100	88	100
Paired elements	0	0	4	0	0	0
Filaments	0	50	58	20	13	7
Variable arrangement	100	100	100	100	100	100
Colony morphology						
Size (mm)						
< 2	100	100	100	100	100	93
2-5	0	0	13	0	0	43
> 5	0	0	0	0	0	4
Flat (effuse)	0	0	0	0	0	0
Raised	20	100	100	100	75	79
Convex	80	100	100	100	100	100
Umbonate	0	0	0	0	0	0
Entire margin	80	100	0	100	25	100
Crenate margin	20	0	100	0	75	0
Erose margin	0	0	0	0	0	0
Fimbriate margin	0	0	0	0	0	0
Transparent	0	0	4	0	0	0
Opaque	100	100	100	100	100	100
White	0	0	25	0	25	36
Off-white to grey	100	100	75	80	75	14
Colour	0	0	0	0	0	64
Rough	0	0	96	0	50	0
Smooth	100	100	4	100	50	100
Mucoid	0	0	0	0	0	7
Growth						
Aerobic						
+	100	100	96	0	88	
++	100	100	17	0	13	0
+++	100	66	0	0	0	7
Anaerobic						
+	100	100	100	100	100	100
++	100	100	100	100	100	100
+++	100	100	100	100	100	100
On Rogosa's agar	0	0	0	0	0	0
At 45 °C	20	0	8	0	13	43
At 37 °C	100	100	100	100	100	100

Table 4 (cont.)

	<i>A. viscosus/ A. naeslundii</i>	<i>A. odontolyticus</i>	<i>A. israelii</i>	<i>Act. meyerii</i>	<i>A. propionica</i>	<i>P. acnes</i>
Biochemical tests						
Aesculin hydrolysis	100	49	100	0	0	0
Gelatin liquefaction	0	0	4	0	0	71
Indole production	0	0	0	0	0	100
Nitrate reduction	100	100	100	100	100	100
Catalase production	40	0	0	0	0	100
Arginine reduction	20	17	96	0	63	100
Voges-Proskauer test	0	0	0	0	0	0
Methyl-red test	40	0	0	100	0	0
H ₂ S production	0	17	4	0	0	0
Starch hydrolysis	40	17	75	0	13	0
Lecithinase production	0	0	0	0	0	0
Acid production from:						
Arabinose	0	17	42	60	0	0
Galactose	80	100	67	100	50	79
Mannose	100	0	100	0	100	93
Xylose	0	83	100	100	0	0
Glucose	100	100	100	100	100	100
Ribose	0	17	92	100	25	50
Lactose	100	100	100	100	13	7
Trehalose	80	0	100	0	100	0
Raffinose	100	0	83	0	63	0
Glycogen	0	0	0	80	0	0
Adonitol	0	0	0	0	0	7
Mannitol	0	0	86	0	100	7
Sorbitol	0	0	8	0	13	64
End-products from glucose						
Acetic acid	100	100	100	100	100	100
Propionic acid	0	0	0	0	100	100
Isobutyric acid	0	0	0	0	0	0
Butyric acid	0	0	0	0	0	0
Isovaleric acid	0	0	0	0	0	86
Valeric acid	0	0	0	0	0	0
Isocaproic acid	0	0	0	0	0	0
Caproic acid	0	0	0	0	0	0
Heptanoic acid	0	0	0	0	0	0
Pyruvic acid	0	0	0	0	0	0
Lactic acid	100	100	100	100	0	7
Oxalacetic acid	0	0	0	0	0	0
Oxalic acid	0	0	0	0	0	0
Methyl malonic acid	0	0	0	0	0	0
Malonic acid	0	0	0	0	0	0
Fumaric acid	0	0	0	0	0	0
Succinic acid	100	100	96	100	100	93

white to grey. They showed limited growth under aerobic conditions. Aesculin and arginine were hydrolysed and nitrate was reduced. They were fermentatively active, and acid was produced from mannitol, mannose, lactose, xylose, trehalose and ribose. Glucose was fermented with the production of acetic, lactic and succinic acids.

Strains of *Act. meyerii* in phenon 3 were all anaerobic. They formed pleomorphic elements including cocco-bacillary and irregularly-formed rods. The colonies were smooth, raised

Table 5. *The mean similarity value, and S.D., for members of the phena A. israelii, Act. meyerii, A. viscosus/A. naeslundii, A. odontolyticus, A. propionica and P. acnes, to their own c.m.o.*

Phenons	<i>A. israelii</i> 2 (n = 24)	<i>Act. meyerii</i> 3 (n = 5)	<i>A. viscosus</i> / <i>A. naeslundii</i> 4A (n = 5)	<i>A. odontolyticus</i> 4B (n = 6)	<i>Ar. propionica</i> 5 (n = 8)	<i>P. acnes</i> 6A (n = 8)
2	92.9 ± 2.3					
3		98.5 ± 1.7				
4A			95.6 ± 3.3			
4B				94.2 ± 2.5		
5					91.6 ± 1.0	
6A						92.0 ± 3.4

and convex, with an entire edge. Most colonies were off-white to grey. They reduced nitrate and gave a positive methyl-red test. Galactose, lactose, xylose and ribose were fermented. Acetic, lactic and succinic acid were produced from glucose.

Strains of *A. viscosus* and *A. naeslundii* of phenon 4A were all facultative anaerobic and able to grow under aerobic conditions. They were irregular rods or cocco-bacilli, often pleomorphic with multiple branching, and produced smooth, convex, off-white to grey colonies with entire or crenate margins. They degraded aesculin and reduced nitrate. Acids were produced from mannose, lactose and raffinose. They fermented glucose with the production of acetic, lactic and succinic acid.

The *A. odontolyticus* strains in phenon 4B had many morphological characters in common with the *A. viscosus/A. naeslundii* strains. They were able to grow aerobically after several subcultures in the laboratory. Nitrate was decomposed and acid formed from galactose and lactose. Acetic, lactic and succinic acids were produced from glucose.

The *Ar. propionica* strains in phenon 5 formed cocco-bacilli to irregular rods with a varying degree of branching; some strains also formed coccoid elements. They produced white or off-white to grey colonies which had many characters in common with *A. israelii*. Aesculin was not hydrolysed but nitrate was reduced. The fermentative pattern of these strains included acid formation from mannose, mannitol and trehalose. Glucose was fermented with the production of acetic, propionic and succinic acid.

The Propionibacterium strains in phenon 6 formed irregular rods and tapered elements with rounded ends in variable arrangements. The colonies were smooth and convex with entire margins. The colonies were opaque, and either white or off-white to grey or pink. The strains showed no distinctive biochemical or fermentative properties. However, all strains fermented glucose with the production of acetic and propionic acid and most strains also produced isovaleric acid. The *P. acnes* strains in phenon 6A were less variable in their biochemical reactions (Table 4). All strains produced catalase and indole, reduced nitrate but failed to hydrolyse aesculin.

Calculation of phenetic variation within the defined phena

In Table 4 are shown the percentages of positive results in the major phena from which the hypothetical 'calculated median organisms' (c.m.o.s) were calculated. Table 5 gives the mean similarity values for the members of the phena to their respective c.m.o. The lowest permissible similarity index to the c.m.o., based on the simple matching coefficient that a candidate strain must possess to be included in the proposed taxospecies of *A. viscosus/A. naeslundii*, *A. odontolyticus*, *A. israelii*, *Act. meyerii*, *Ar. propionica* and *P. acnes*, can be

calculated. The members of the phena showed consistently high similarity values to their respective c.m.o.s (Table 6). The new isolates, which in the numerical analysis appeared outside the major phena, exhibited similarity values to the created c.m.o.s falling outside a 5 % range from the permissible low limits.

Reproducibility of results

An additional statistically designed experiment was performed in order to afford a check on experimental error in performing tests. The total array of tests was done in duplicate on the 26 new isolates from dental plaques representing members of all the defined phena. These tests were carried out on two separate occasions, employing the same medium, techniques and conventions for scoring. Twenty-eight of the tests gave identical results on both occasions in all the 26 strains. The least reproducible tests were on some morphological character states, anaerobic growth at 45 °C, hydrolysis from arginine, methyl-red test, H₂S production, starch hydrolysis and production of acid anaerobically from 1 % (w/v) raffinose. If the tests with identical results were excluded, the probability (*p*) of an erroneous result averaged 3.12 %, equal to an observed value of S_{SM} of around 94 % between sub-cultures of the same strains. The similarity value between the identical strains 1452/40-41 (Holm) and VPI3258 was 95.6 %, between ATCC12104 and NCTC10301 94.2 %, and between ATCC17982 and NCTC9931 was 92.3 %, as calculated by the simple matching coefficient (S_{SM}).

Gas chromatographic analyses by two instruments with different operating conditions on the same sample gave identical results with respect to the qualitative interpretation of the metabolic end-products produced in glucose fermentation.

Identification by immunofluorescence

In direct immunofluorescence, all new isolates and reference strains which in the numerical analysis were grouped within the phena of *A. israelii*, *Act. meyerii*, *A. viscosus*/*A. naeslundii*, *A. odontolyticus*, *Ar. propionica* and *P. acnes*, exhibited positive immunofluorescent reactions with species-specific conjugates to the type strains of the respective species (Table 6). All other strains were negative to these conjugates in immunofluorescence, but there was one aberrant result – the indole-producing strain 87, which appeared peripheral to the *P. acnes* cluster, showed a positive reaction with anti-*P. acnes* serotype 1 (NCTC737) serum.

The strains in the phena of *A. viscosus*/*A. naeslundii*, *A. odontolyticus* and *Ar. propionica* provided no correlation between phenotypic characteristics and serotype. Strains allocated to the phenon of *A. viscosus*/*A. naeslundii* showed positive immunofluorescent staining to *A. viscosus* serotype 1 (ATCC15987), *A. viscosus* serotype 2 (ATCC19246) or *A. naeslundii* (ATCC12104) antisera.

Diagnostic table

Each defined phenon was examined for distinguishing features that could be weighted for identification. By comparing the frequencies of occurrence for the total array of characters within each defined phenon a diagnostic table could be constructed for the differentiation and identification of *A. viscosus*/*A. naeslundii*, *A. odontolyticus*, *Act. meyerii*, *A. israelii* and *Ar. propionica* from the related propionibacteria and bifidobacteria (Table 7). In the case of propionibacteria, characters were also devised for the identification of *Propionibacterium acnes*.

Table 6. The similarity values to the c.m.o.s of the major phena *A. israelii*, *Act. meyerii*, *A. viscosus* | *A. naeslundii*, *A. odontolyticus*, *Ar. propionica* and *P. acnes*, and the immunofluorescence reactions with conjugates to type-strains of these species for strains clustering in these phena according to the overall similarity

	S-value to the c.m.o. of	Immunofluorescent reactions with conjugates to
K. HOLMBERG AND C.-E. NORD		
ATCC11828		
NCTC737		
WVU346		
ATCC14157		
WVU482		
ATCC17982		
ATCC12104		
ATCC19246		
ATCC15987		
VPI8617	+	
WVU307	+ + +	
ATCC12103	+ + + + + + + + + - + + + + + +	
<i>P. acnes</i> (6A)	57·9 60·8 60·8 59·4 59·4 59·4 60·8 59·4 59·4 66·6 60·8 57·9 65·2 63·7 62·3 62·3 60·8 59·4 63·7 62·3 62·3 57·9 62·3 62·3	
<i>Ar. propionica</i> (5)	76·8 79·8 79·7 81·1 78·2 75·3 73·9 75·3 75·3 79·7 79·7 73·9 81·1 79·7 81·1 78·2 73·8 84·0 82·6 81·1 81·1 73·9 78·2 72·4	
<i>A. odontolyticus</i> (4B)	75·3 75·3 75·3 76·8 73·9 73·9 69·5 73·9 73·9 69·5 72·4 72·4 79·7 79·7 79·7 76·8 75·3 79·7 82·6 79·7 76·8 72·4 73·9	
<i>A. viscosus</i> / <i>A. naeslundii</i> (4A)	75·3 75·3 75·3 76·8 71·0 71·0 69·5 71·0 71·0 75·3 75·3 69·5 76·8 75·3 79·7 73·9 82·6 81·1 76·8 76·8 69·5 76·8 68·1	
<i>Act. meyerii</i> (3)	66·7 72·5 69·6 68·1 68·1 62·3 63·8 65·2 68·1 69·6 69·6 63·8 71·0 69·6 68·1 71·0 82·6 72·5 65·2 71·0 63·8 68·1 68·1	
<i>israelii</i> (2)	94·2 91·3 94·2 92·7 92·7 92·7 88·4 95·6 95·6 88·4 88·4 94·2 91·3 95·6 94·2 92·7 95·6 94·2 89·8 95·6 91·3 95·6 89·8	
Strain no.	12103* 307* A555/73 A752/73 104/45-46 169/45-46 175/45-46 1061/40-41 1452/40-41 1654/40-41 1919/40-41 2129/40-41 8047 A18 A9 A26 66 69 71 75 76 78 Bel5	
Phenon	2	3

Table 7. *Differential characters for A. naeslundii/A. viscosus, A. odontolyticus, A. israelii, Act. meyerii, Ar. propionica and P. acnes*

Differential characters	<i>A. naeslundii/A. viscosus</i>	<i>A. odontolyticus</i>	<i>A. israelii</i>	<i>Act. meyerii</i>	<i>Bifidobacterium</i> spp.	<i>Ar. propionica</i>	<i>P. acnes</i>	Other species of <i>Propionibacterium</i>
Aerobic growth*	+	D	—	—	—	—	—	—
End-products from PYG broth	A, L, S	A, L, S	A, L, S	A, L, S	A, L, (S)	A, P, S	A, P, (IV), S	A, P, (IV, L, S)
Aesculin hydrolysis	+	D	+	—	+	—	—	D
Indole production	—	—	—	—	—	—	+	—
Nitrate reduction	+	+	+	+	—	+	+	D
Catalase production	D†	—	—	—	—	—	+	D
Acid from mannitol	—	—	D	—	D	—	—	—
Acid from xylose	—	D	+	+	D	—	—	D
Acid from arabinose	—	D	D†	D	D	—	—	D
Acid from sorbitol	—	—	—	—	—	D	D§	D

A, acetic acid; L, lactic acid; S, succinic acid; P, propionic acid; IV, isovaleric acid. Acid products sometimes produced are given in parentheses. +, Positive; —, negative; D, doubtful.

* Heavy growth (with or without CO₂).

† *Actinomyces viscosus*, +; *A. naeslundii*, —.

‡ *Actinomyces israelii* serotype 1, +; *A. israelii* serotype 2, —.

§ *Propionibacterium acnes* serotype 1, +; *P. acnes* serotype 2, —.

DISCUSSION

Most new isolates of facultative anaerobic and obligate anaerobic Gram-positive filamentous and/or diphtheroidal organisms and the reference strains of predictor genera were distributed in one of six clusters. By comparison with the current taxonomic views (Prévot, 1966; Pine, 1970; Cross & Goodfellow, 1973) of the actinomycetes and the allocation of the included marker strains, the taxonomic rank of these clusters would be appropriately accepted as equivalent to generic taxa of *Actinomyces*, *Arachnia*, *Actinobacterium*, *Bifidobacterium* and *Propionibacterium*. These defined clusters (phena) appeared to be valid. There was good consonance of the clusters formed by the single linkage and by the unweighted average linkage cluster methods on the resemblance coefficients, and the homogeneities of the phena were high as measured by the intra-group mean similarity values.

There is always a danger with numerical classification that taxonomic groups are influenced by differences in growth rates and cultural conditions (Sneath, 1968). This is particularly true with the facultative anaerobic *Actinomyces* species and the anaerobic *A. israelii* and *Ar. propionica* which differ in the rate of growth (Pine, 1970). The differences observed between the phena of these organisms appeared not to be due to unequal growth rates. The uniform mean vigour values of the phena indicated that comparison was made between clusters that were equally vigorous, i.e. their ability to carry out varied metabolic activities was about the same as measured by the number of positive reactions in the tests employed. Reading the tests at a specified time, after exactly five days of incubation at 37 °C for fast-growing as well as slow-growing strains, had the effect of reducing the introduction of phenetic dissimilarities due to unequal growth rates, since it is uncommon for positive tests to revert to negative on continued incubation. Similar considerations apply to an accurately-controlled atmosphere for incubation, since variations in the degree of anaerobiosis might have the effect of changing metabolic activity. This difficulty was overcome by assembling all data on phenotypic characters from tests performed under strict anaerobiosis. On the other hand, the work by Melville (1965) with facultative anaerobic actinomycetes suggests that the phenetic resemblances are much the same, whether they are grown aerobically or anaerobically.

The influence of the reproducibility of tests on similarity values was recently discussed by Sneath & Johnson (1972). The estimated probability of an incorrect test averaged 3 % over the set of tests employed in the present study, if the time of reading was specified as 5 days of incubation at 37 °C. This amount of experimental error in the similarity values has in practice been found acceptable in numerical taxonomic studies (Sneath & Johnson, 1972; Sneath, 1972). The level of similarity of 93 to 96 %, estimated between the replicate strains, is a confirmation of this opinion. However, the significance of the observed differences between the defined clusters has to be judged against the confidence limits of the similarity values, estimated as the sum of the experimental error and the sampling error of the 90 tests employed.

Among the major phena two clusters of *Actinomyces* strains equivalent to generic rank were recognized. One cluster contained the strains of *A. israelii* and the other the facultative anaerobic *Actinomyces* species including the representatives of *A. viscosus*, *A. naeslundii* and *A. odontolyticus*. The results throw doubts on the current taxonomic theory of a single genus *Actinomyces*, containing both the anaerobic and the facultative anaerobic *Actinomyces* strains. The anaerobic *A. israelii* cluster constituted a tight taxonomic entity quite distinct from other *Actinomyces*. Prévot (1961) considered *A. bovis* and *A. israelii* to be sufficiently distinct to receive different generic names. He placed *bovis* in the genus *Actinomyces* and

israelii in the genus *Actinobacterium*. The relatively simple carbohydrate component pattern of *A. israelii* walls compared with those of *A. bovis*, *A. naeslundii*, *A. viscosus* and *A. odontolyticus* (Boone & Pine, 1968; DeWeese, Gerencser & Slack, 1968; Pine, 1970) is also evidence of heterogeneity. Further work is needed to determine whether the *A. israelii* group deserves recognition as a separate genus within the family Actinomycetaceae.

Within the *Actinomyces israelii* phenon a second variant that failed to ferment arabinose was discernible as a distinct serotype by immunofluorescence. A second type of *A. israelii* is well documented and was first described by Holm (1930). The principle differences were that these variant strains gave viscous growth in liquid medium and failed to ferment arabinose (Holm, 1948; Brock & Georg, 1969*b*; Cummins, 1970). The separation of two variants of *A. israelii* has also been confirmed by serological methods (Lambert *et al.* 1967; Slack *et al.* 1969; Brock & Georg, 1969*a*; Holmberg & Forsum, 1973; Holmberg, Nord & Wadström, 1975*a, b*) and by wall analysis (Cummins, 1970).

The facultatively anaerobic *Actinomyces* phenon was separated into two sub-generic groups which appeared to be acceptable taxospecies. One subgroup included the named strains of *A. viscosus* and *A. naeslundii*. It is well known that these organisms are phenetically close species, and this has recently been discussed by Holmberg & Hallander (1973). Our present phenetic estimates on *A. viscosus* and *A. naeslundii* agree well with the results from this earlier numerical taxonomic work. They also confirm the close phenetic resemblance between these organisms under strictly anaerobic conditions in pre-reduced media. The type strains of *A. odontolyticus* (Batty, 1958) were found in the other sub-generic group of the facultative anaerobic *Actinomyces* phenon. The existence of a second serotype of *A. odontolyticus* (Slack & Gerencser, 1970*a*) was verified by immunofluorescence, although it was not correlated to any specific phenetic properties. The recovery of the only strain of *A. bovis* that we studied in the facultative anaerobic *Actinomyces* cluster is consistent with the results of the numerical taxonomic study by Melville (1965) who recovered the type strains of *A. odontolyticus* in the *A. bovis* cluster.

The five strains received as *Act. meyerii* (Prévot, 1938, 1961, 1966, 1972) appeared to represent a phenotypically distinct group. Bouisset *et al.* (1968) noted that strains of *Act. meyerii* had guanine-cytosine base ratios that fall in the narrow range of 64 to 67, close to those of *Actinomyces (Actinobacterium) israelii* and *A. bovis*. The strains of *Act. meyerii* examined in the present study were distinct by immunofluorescence, which provides further evidence for the segregation of these bacteria as a separate taxon. They appeared close to the *Actinomyces* so that they might be best associated with the Actinomycetaceae. Phenetically, they were closer to the facultative anaerobic *Actinomyces* than to *A. israelii*. This difference between *Act. meyerii* and *A. israelii* was not due to difference in the metabolic activity, since clustering on pattern results showed the same relationship between these organisms. The species *Act. abscessum*, of which we had only a single representative, did not fall into any defined cluster, which suggests that this species should not be allied with the Actinomycetaceae. The genetic study by Bouisset *et al.* (1968) supports this.

The genus *Arachnia* was set up by Pine & Georg (1969) to accommodate propionate-producing bacteria with diaminopimelic acid in their wall, previously classified as *Actinomyces propionicus* (Pine & Hardin, 1959; Buchanan & Pine, 1962). The phenotypic data presented here indicate that the *Arachnia* strains form a recognizable taxon, phenetically distinct from the other generic phenon. This confirms serological (Georg *et al.* 1964; Gerencser & Slack, 1969; Holmberg & Forsum, 1973) and genetic (Johnson & Cummins, 1972) evidence which indicates that the strains of *Arachnia* form a reasonably homogeneous group. The differentiation of two variants of *Ar. propionica*, previously proposed on the

basis of wall composition and serological reactions, was confirmed by immunofluorescence.

The existence of a distinct generic phenon of bifidobacteria appeared to be justified on phenetic estimates. The separation of *Bifidobacterium* strains as a distinct group would support the recognition of bifidobacteria as a separate genus classified in the family Actinomycetaceae (*Bergey's Manual of Determinative Bacteriology*, 1974).

However, the *Bifidobacterium* cluster as it stands probably represents a heterogeneous mixture not clearly definable by the present taxonomic estimates. The recognition within the cluster of minor groups and single strains of named bifidobacteria and new isolates which joined at rather low similarity levels confirmed the existence of multiple species and species variants of human bifidobacteria (Dehnert, 1957; Reuter, 1963, 1971; Mitsuoka, 1969; Scardovi *et al.* 1971). The data provide evidence for the existence of an additional group of mannitol-fermenting oral bifidobacteria, similar to the unassigned 'dentium' group of bifidobacteria proposed by Scardovi *et al.* (1971), previously isolated from the human mouth by Beerens, Gérard & Guillaume (1957) and designated as *B. dentium* (Scardovi & Crociani, 1974).

The type strain of *Bifidobacterium eriksonii* (Georg *et al.* 1965) was allocated to the *Bifidobacterium* cluster. The strain was fermentatively similar to the bifidobacteria. The inclusion of this organism in the *Bifidobacterium* genus is strongly supported on this ground (*Anaerobe Laboratory Manual*, 1973) although *B. eriksonii* has been reported to have the same qualitative wall component pattern as *A. israelii* serotype 1 (Boone & Pine, 1968). The type strains of *B. bifidum* (ATCC15696) were not included in the phenon of *Bifidobacterium* and showed very little phenetic affinity with the other bifidobacteria.

A sufficiently distinct cluster was formed of *Propionibacterium* strains to warrant their designation as a separate genus. There has been much confusion concerning the genus *Propionibacterium* and disagreement on what should be considered valid species (Prévot, Turpin & Kaiser, 1967; Moore, Holdeman & Cummins, 1968; Zeirdt, Webster & Rude, 1968; Reid & Joya, 1969). Recently, on the basis of nucleic-acid homology studies, Johnson & Cummins (1972) designated three major groups, *P. acnes*, *P. avidum* and *P. granulosum*. *Propionibacterium acnes* was further separated into two distinct types, based on the carbohydrate composition of the walls. In the present study the named strains of *P. acnes* fall into a sub-generic group which can be equated with a taxospecies. The boundary of this cluster was, however, rather diffuse. It is possible that strains of this cluster overlap with related species to a small extent or are phenetically intermediates, which would corroborate the reported phenetic heterogeneity of this species (Brzin, 1965; Puhvel, 1968; Voss, 1970; Pulverer & Ko, 1973). The strain D34, received from Dr Voss as *Corynebacterium acnes* group 2 (Voss, 1970) was phenetically associated with *P. acnes*. The position is, however, not entirely clear because the strain showed negative reactions with mono-specific conjugates against both the serotypes of *P. acnes*, but positive immunofluorescence with mono-specific conjugate against the type strains of *P. granulosum* (ATCC25564). This type strain of *P. granulosum* appeared to be phenetically more similar to *P. acnes* than to *P. avidum*. The relationships between these species do not correlate consistently with genetic data, wall carbohydrate pattern (Johnson & Cummins, 1972) and serological data obtained by immunofluorescence (Holmberg & Forsum, 1973) on these organisms.

It is worth noting the taxonomic relationships found between the defined phenons of *Actinomyces*, *Arachnia*, *Propionibacterium* and *Bifidobacterium*, since the question of the relationships of actinomycetes, propionibacteria and lactobacilli (bifidobacteria) has always been a vexatious problem (Puntoni, 1937; Prévot, 1938; Pine, 1970; Poupard, Husain &

Norris, 1973). It appeared that the *Actinomyces*, including *Actinobacterium meyerii*, showed a greater phenetic affinity to bifidobacteria than to propionibacteria. Pine & Howell (1956) discussed the relationship of bifidobacteria to *Actinomyces*; they considered a major difference to exist between *Actinomyces* and the bifidobacteria, on the basis of the difference in the amounts of acetic and lactic acid produced. Pine (1970) concluded, in agreement with Snyder *et al.* (1967), that bifidobacteria are not serologically related to *Actinomyces*.

A somewhat higher average inter-group similarity to the *Actinomyces* clusters than to the *Propionibacterium* cluster suggests that the *Arachnia* strains should be classified within the Actinomycetaceae. The *Arachnia* strains, however, have several important characters in common with the propionibacteria, which suggest that they are intermediates between the families Actinomycetaceae and Propionibacteriaceae. *Arachnia* resemble propionibacteria in production of propionic acid and in the amino acid pattern of their wall peptidoglycan, but showed a low genetic similarity with these bacteria in DNA pairing studies (Johnson & Cummins, 1972).

The numerical taxonomy resulted in natural groups which allowed the construction of a diagnostic table for the differentiation of *A. israelii*, *A. viscosus*, *A. naeslundii*, *A. odontolyticus*, *Act. meyerii*, *P. acnes* and *Ar. propionica* strains from the related bifidobacteria and propionibacteria. However, the present phenotypic information did not enable differentiation of other propionibacteria and bifidobacteria into species, and insufficient data were obtained to devise phenotypic characteristics for eubacteria and anaerobic lactobacilli. It was apparent that no single test could be selected that would differentiate *Actinomyces* and *Arachnia* from similar organisms or distinguish between different *Actinomyces* species. Identification of an unknown organism as an *Actinomyces* or *Arachnia* species has to be made on the results of a series of tests to provide specific means of identification. The diagnostic table was generated by means of a simultaneous strategy of identification (Sneath, 1972) giving no character an infinite weight. Other non-sporeforming Gram-positive anaerobes may have the same reactions in two or three of the selected tests, but we have not found this set of reactions in a strain of eubacteria or lactobacilli.

In the interpretation of the value of morphological characters for identification, no character was found to be sufficiently discriminative and mutually exclusive to justify its inclusion in a diagnostic table. However, these organisms have morphological features which, while not unique, when taken together might assist a practised eye to distinguish between genera (Moore & Holdeman, 1970). Many of the morphological characters that might be considered typical for these organisms are subjective, and all are influenced by temperature, incubation time and the nutritional qualities of the basal medium (see, for example, Beerens *et al.* 1957; Sundman & Björkstén, 1958; Slack & Gerencsér, 1970*b*). Thus, they are variable because many of the factors that affect them are difficult to control.

Micro-colony morphology is a useful characteristic for recognizing an *Actinomyces* (Slack *et al.* 1969; Slack & Gerencsér, 1970*b*) and also for differentiating the species. Although the presence of a filamentous micro-colony may be indicative of an *Actinomyces*, we have found such colonies to be extremely difficult to see and the dominant forms are diphtheroids, bifid, or even rod shaped. Since this property cannot be considered as a unit character acceptable for numerical taxonomy it was omitted from the analysis.

The tests for optimal growth under aerobic and anaerobic conditions were included as an initial basis for the differentiation of this group of organisms in accordance with the recommendations of the *Subgroup on the Taxonomy of Micro-aerophilic Actinomycetes* (1968). The anaerobic condition allowed good growth of all strains. Although strict anaerobiosis was an obligate requirement for *Bifidobacterium* species, it was not completely

exclusive for *Actinomyces*, *Arachnia* or *Propionibacterium* species; some of these bacteria grow under aerobic conditions after continued laboratory culture, and the addition of CO₂ stimulates the aerobic growth of *Actinomyces* species (Rosebury, 1944; Buchanan & Pine, 1965; Pine, 1970), being metabolized at a substrate level (Pine & Howell, 1956). As judged by oxygen tolerance these organisms are to be characterized as moderate anaerobic organisms (Loesche, 1969).

Analysis of the metabolic end-products from glucose fermentation by the actinomycetes under strict anaerobic condition in the presence of added CO₂ was found to be valuable for the differentiation of genera. The profiles of the gas-liquid chromatograms for volatile and non-volatile fatty acids were highly reproducible in repeated tests on the same strain and identical results were obtained when different instruments were used for the tests. The chromatograms are characteristic for *Actinomyces*, *Arachnia*, *Bifidobacteria* and *Propionibacteria* with respect to the production of acetic, propionic, isovaleric, lactic and succinic acids. Propionate formation was found to be characteristic for the *Arachnia* and the *Propionibacterium* strains; no propionic acid was produced by *Actinomyces* and *Bifidobacterium* strains. At this point our results are not in agreement with those of Li & Georg (1968), who demonstrated trace amounts of propionic acid in the chromatographic analysis of volatile acids in the medium in which *A. israelii* and *A. naeslundii* had grown, but are consistent with those obtained from gas chromatographic analysis by Buchanan & Pine (1962).

The information provided by gas chromatographic examination on a qualitative basis does not allow a proper differentiation between *Actinomyces* and bifidobacteria. However, a simple approximate estimation of the amounts of the acids produced, yielded information quite useful for their differentiation. Bifidobacteria produce acetic and lactic acid as their major metabolic products in the approximate ratio of 1.5 to 1.0, and trace amounts of succinic acid were occasionally detected, whereas the *Actinomyces* produce lactic acid and succinic acid as their major non-volatile acids, and acetic acid as the only major volatile acid. Certain points pertain to the effects of CO₂ and the degree of anaerobiosis in the interpretation of these assays. Qualitative and quantitative changes in fermentation capacity of the *Actinomyces* are observed under anaerobic and aerobic conditions and in the presence or absence of CO₂. The strains of *Actinomyces* incorporate added CO₂, with a high production of succinic acid and smaller amounts of lactic acid, when glucose is fermented anaerobically (Rosebury, 1944; Pine & Howell, 1956; Buchanan & Pine, 1965). Strains of *Actinomyces*, which can grow in aerated media in the presence of CO₂, ferment glucose with the formation of only acetic acid (Buchanan & Pine, 1965; Pine, 1970). However, under the anaerobic conditions we employed there is probably sufficient constancy in these characters to differentiate between *Actinomyces* and bifidobacteria.

The assays on acid end-products could also be useful for differentiating *Actinomyces* and bifidobacteria from other related anaerobes, such as lactobacilli and eubacteria. Obligate anaerobic species of lactobacilli produce only lactic acid as a major product from glucose fermentation (Moore & Holdeman, 1970, 1973). The definition of the genus *Eubacterium* has been emended to include organisms that do not produce the following as major products of fermentation: propionic acid; lactic acid alone; acetic and lactic acids together but with the amount of acetic acid exceeding that of lactic acid (Moore & Holdeman, 1973). Most species in this genus produce butyric and other acids; some species mainly produce acetic acid.

Some biochemical tests were used as additional tools in the differentiation of genera and species. Hydrolysis of aesculin was a constant feature of all *Actinomyces* species, except for *A. odontolyticus*. This property helped to differentiate *Actinomyces* from *Arachnia pro-*

pionica and *Propionibacterium acnes*, but not from other propionibacteria and bifidobacteria. The anaerobic strains of *Actinobacterium meyerii*, which produce metabolic end-products of glucose fermentation similar to those of *Actinomyces* strains, could be separated from these organisms by their failure to hydrolyse aesculin. No strains of bifidobacteria reduced nitrate. This character merits being a key feature of bifidobacteria (Scardovi *et al.* 1971; Reuter, 1971) and separates them from the *Actinomyces* and *Arachnia*. In other reports (Slack & Gerencser, 1970c; Slack *et al.* 1969; Brock & Georg, 1969a; Georg, 1970; Moore & Holdeman, 1970) nitrate reduction has not been a definite property of *A. israelii*, but all strains assigned as *A. israelii* in the present study reduced nitrate to nitrite in the oxygen-free indole-nitrite medium. Several factors may be responsible for these contradictory results, e.g. the capacity for assimilatory and respiratory nitrate reduction in aerated and pre-reduced media. The activity and production of nitrate reductase is suppressed in aerated media (Payne, 1973), and since *Actinomyces israelii* grows in limited aerated media, assays of nitrate reductase activity in such media might not have given detectable levels of nitrite. Production of indole was an exclusive character of all strains of *P. acnes*. The indole test proved to be useful for separating *P. acnes* from the *Actinomyces* and *Arachnia* as well as from other propionibacteria and bifidobacteria on which it is a latent 'contaminant'. An inverse correlation between indole production and nitrate reduction by *P. acnes* strains (Moore & Holdeman, 1973) was not confirmed in the present work. The absence of catalase in *Arachnia* and *Actinomyces*, except for *A. viscosus*, serves to separate these genera from most members of the genus *Propionibacterium*.

Acid formation from carbohydrates is not a truly independent characteristic for anaerobes, as variations occur in the carbohydrate fermentation under different degrees of anaerobiosis and a fall in pH value accompanies high CO₂ tension (Watt, 1973; *Anaerobe Laboratory Manual*, 1973). Nevertheless, from the results we concluded that some reactions were of value in identification, provided that the carbohydrate concentration in the fermentation medium was reduced to 1 % (w/v) and that phosphate (1.5 %, w/v) was used as a buffer. The characteristics listed in the diagnostic table indicate reactions of cultures in which there was excellent growth after 5 days or more of incubation at 37 °C. Acid production from mannitol was a property shared by *Ar. propionica* and some *A. israelii*, but not by other *Actinomyces* species. The reaction with xylose differentiated between *Ar. propionica* and *A. israelii*. *Actinomyces israelii* serotype 2 failed to ferment arabinose, and sorbitol-fermenting strains of *P. acnes* belonged to serotype 1 of this species.

In practice, bacterial specimens sampled and transported in pre-reduced media are cultivated in PRAS PYG roll streak-tubes or on freshly prepared pre-reduced PYG medium in plates, inoculated in a GasPac atmosphere. Colonies of Gram-positive non-sporeforming pleomorphic rods from primary anaerobic tubes or plates are subcultured to determine purity and tested for sensitivity to O₂ by culture on aerated PYG medium. Pure anaerobes are cultured on PRAS broth in CO₂-gassed tubes in duplicate. Five-day-old cultures are subjected to gas chromatographic analyses for end-products from glucose fermentation, and to a series of biochemical tests. For this purpose one PRAS PYG broth culture is washed under continuous anaerobiosis in pre-reduced salt solution and a bacterial suspension with an extinction of approximately 0.2 at 540 nm is prepared. This suspension is aspirated in a gassed-out syringe and portions are inoculated into tubes containing the differential media: aesculin, indole-nitrite and fermentation bases with mannitol, xylose, arabinose and sorbitol. Alternatively, the application of immunofluorescence in identification of the species of *Actinomyces* and *Arachnia* is as efficient (Holmberg & Forsum, 1973) as determination of biochemical characteristics, as well as being simpler and quicker.

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