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A Numerical Taxonomic Study of Members of the *Actinomycetaceae* and Related Taxa

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Two hundred and twenty-two representatives of the *Actinomycetaceae* and related taxa, including reference cultures, received strains and fresh isolates, were tested using 124 unit characters. The data were examined using numerical taxonomic techniques with various coefficients and average linkage clustering; the variation in composition of the clusters obtained from the different coefficients was slight. Most species included in the study formed discrete phena, which exhibited good differential characters. *Actinomyces israelii* was particularly well-defined and *A. naeslundii* and *A. viscosus*, although grouping together, did show divergence in several test results. *Actinomyces bovis* was initially linked with representatives of genera other than *Actinomyces*, these being *Bifidobacterium*, *Corynebacterium pyogenes* and *Erysipelothrix rhusiopathiae*. The strains of *Bacterionema matruchotii* and *Rothia dentocariosa* formed tight distinct clusters associated only at low similarity levels with other members of the family *Actinomycetaceae*. The *Arachnia propionica* phenon, although well-defined, appeared to consist of two subclusters which could be assigned to serotypes 1 and 2. Tests which may be useful in identification are tabulated and the relationships between the various taxa are discussed.

INTRODUCTION

Gram-positive, non-spore-forming bacteria which are predominantly diphtheroid in shape but tend to form branched filaments, and possess a fermentative carbohydrate metabolism, fulfil the basic requirements for being placed in the family *Actinomycetaceae* as defined in the 8th edition of *Bergey's Manual of Determinative Bacteriology* (Slack, 1974). However, although usually highly distinctive of *Actinomycetaceae*, similar properties may well be found in members of other families, e.g. *Propionibacteriaceae* (Moore & Holdeman, 1974; Schaal *et al.*, 1980). Therefore, classification and especially identification of fermentative actinomycetes still present problems despite the contributions of modern taxonomic techniques. Methods such as cell wall, lipid and acid end-product analyses are useful for defining fermentative actinomycetes at the genus level (Schaal *et al.*, 1980; Schaal & Schofield, 1981 *a*, *b*; Slack & Gerencser, 1975). However, the definition of most of the actinomycete species, especially in the genus *Actinomyces*, is still unsatisfactory.

Morphological resemblances played an important role in previous classification systems. However, cellular and colonial morphology may vary considerably within, as well as between, different species, and thus may not draw clear lines of demarcation between organisms of different taxonomic affiliation (Schaal *et al.*, 1980; Schaal & Pulverer, 1981). Physiological and biochemical tests which are frequently used in identification of fermentative actinomycete species are difficult to standardize (see Slack & Gerencser, 1975).

As yet, genetic data (Coykendall & Munzenmaier, 1979; Johnson & Cummins, 1972) have contributed little towards the clarification of this area of bacterial taxonomy. The techniques

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for the determination of GC ratios and DNA:DNA reassociation are relatively complex and time-consuming and, therefore, are usually performed on only a few strains. If these strains have been previously incorrectly classified, interpretation of the results is difficult.

Only a few numerical taxonomic studies of *Actinomycetaceae* have been reported (Holmberg & Hallander, 1973; Holmberg & Nord, 1975; Melville, 1965). In these surveys there was considerable reliance on colonial and cell morphology and many of the physiological tests included were those initially designed for aerobic bacteria. A recent limited numerical study of 48 strains (Fillery *et al.*, 1978) included serological and cell wall information and little morphological data. This separated strains of *Actinomyces naeslundii* and *Actinomyces viscosus* into distinct groups, but produced few physiological characters useful for identification.

We have therefore undertaken a numerical taxonomic study of the *Actinomycetaceae* and related taxa in order to improve the classification of these organisms and also to provide rapid and reliable tests for identification.

METHODS

Strains and cultivation. The bacteria investigated were 107 reference cultures and received strains (Table 1), together with 115 fresh isolates. The latter mostly originated from pus or sinus discharge, and had been tentatively identified using fermentation tests (Minitek system; Becton Dickinson and Co., Heidelberg, F.R.G.) and indirect immunofluorescence (Schaal & Pulverer, 1973). The strains were lyophilized and working stocks were maintained in Fortner plates (Fortner, 1928, 1929) on CC medium (Heinrich & Korth, 1967). Before testing, the strains were grown for 7 d on brain heart infusion agar (BHIA; Oxoid, CM225), either aerobically or anaerobically in GasPak jars (Becton Dickinson), and checked microscopically for purity at $\times 100$ magnification (Schaal & Pulverer, 1981); at the same time their Gram reaction was determined by Hucker's modification (Cowan & Steel, 1975). Incubation for all tests was done in the appropriate gaseous atmosphere and, unless otherwise stated, at 37 °C.

Gaseous growth conditions. All strains were tested for their oxygen and/or carbon dioxide requirements using the method described by Slack & Gerencser (1975).

Catalase. Bacteria from 7 d cultures on BHIA were tested with 3% (v/v) H_2O_2. Anaerobically grown strains were left exposed to air for 1 h prior to testing.

Cytochrome oxidase. The method of Deibel & Evans (1960) was used on cultures grown for 7 d on BHIA.

Deoxyribonuclease. Plates of DNAase medium (Oxoid, CM321) were inoculated, incubated for 7 d and then examined for a transparent zone around the bacterial growth after flooding with 1 M-HCl.

Hyaluronidase and chondroitin sulphatase. The basal medium was BHI broth (BHIB) plus 10 g agar l^{-1} (Difco Noble). To this molten cooled base, hyaluronic acid or chondroitin (400 $\mu g\ ml^{-1}$) was added from a filter sterilized solution. Bovine serum albumin (1%, w/v) was added with constant stirring and then 10 ml of each medium was poured on to a 10 ml tap water agar base. After 14 d incubation, the plates were flooded with 2 M-acetic acid, left for 15 min and then examined for clear zones around the bacterial growth.

Casein, gelatin, starch and Tween hydrolysis, and egg yolk reaction. Various basal media for the different tests were investigated (Slack & Gerencser, 1975; Cowan & Steel, 1975; Holdeman & Moore, 1972) as well as a single base such as BHIA or nutrient agar. However, problems of reproducibility of the results with complex media, or non-growth of some cultures on simpler media were encountered. The basal medium (BM) found to be most suitable was double strength Antibiotics 3 broth (Oxoid, CM287) with 15 g agar l^{-1} (Oxoid, no. 4). For casein plates, 200 ml of 10% (w/v) skim milk (Oxoid, L37) was added to 800 ml of BM at pH 7·2; these plates were examined for clearing at 7 and 14 d after inoculation. Starch was added to BM at a concentration of 0·5% (w/v); after 14 d incubation the medium was flooded with Lugol's iodine solution and examined for a colourless zone around the bacterial growth. Gelatin was added to BM to give a final concentration of 0·4% (w/v) at pH 7·4; 14 d after inoculation the plates were flooded with Frazier's reagent (Cowan & Steel, 1975) and examined for clear areas indicating a breakdown of gelatin. Tweens 20, 40, 60 or 80 (1%, v/v; Merck) together with $CaCl_2.2H_2O$ (0·1 g l^{-1}, from a 10%, w/v, filter-sterilized solution) were added to BM at pH 7·2; crystal formation in the Tween media was recorded at 7, 14 and 21 d. Sterile egg yolk emulsion (Oxoid, SR47), previously heated to 50 °C, was added to cool sterile BM to give a final concentration of 5% (v/v); plates were examined after 7 and 14 d incubation for precipitation and for any lipolytic activity.

Tyrosine, guanine, hypoxanthine, xanthine and adenine hydrolysis. Nutrient broth (Difco, 0003-01) at pH 7·2 with 20 g agar l^{-1} (Oxoid, no. 4) was used as the basal medium. Each of the substances was added to give a final concentration of 0·5% (w/v); 10 ml of each test medium was poured on to a tap water agar base, taking care to

Table 1. *Strains and reference cultures, listed in the order in which they occur in Fig. 1*

Cluster	Sub-cluster	Strain name/number*	Origin
1	a	*Actinomyces israelii* ATCC 10048 (1)	Human pleural fluid
		322 HIK A 492/78 (1)	Pus, extraoral incision
		68 HIK A 667/75	Tissue from intraoral incision
	b	2 HIK A 490/75 (1)	Chronic osteomyelitis
		7 HIK A 722/75 (1)	Blood
		20 HIK A 654/75 (2)	Pus
		3 HIK A 545/75 (1)	Pus and blood, sublingual abscess
		4 HIK A 549/75 (1)	Pus, extraoral incision
		9 HIK A 77/76 (1)	Pus
		5 HIK A 551/75 (1)	Granuloma, extraoral incision
		6 HIK A 658/75 (1)	Blood
		8 HIK A 23/76 (1)	Pus
		17 HIK A 595/75 (2)	Pus, extraoral incision
		23 HIK A 704/75 (2)	Pus, subcutaneous abscess
		19 HIK A 624/75 (2)	Pus
		18 HIK A 616/75 (2)	Pus
		A. israelii 'Wurm' strain (2)	Pus
		21 HIK A 660/75 (2)	Pus
		22 HIK A 704/75 (2)	Pus
		A. israelii ATCC 12102 (1)	Brain abscess, human
		16 HIK A 537/75 (2)	Pus, extraoral lesion
		48 HIK A 578/75	Pus, subcutaneous submandibular process
		26 HIK A 28/76	Pus and blood, cheek abscess
		27 HIK A 31/76	Pus and blood, extraoral lesion
		A. israelii ATCC 23860 (2)	Human parotid abscess
		A. israelii ATCC 10049 (1)	Human actinomycosis
		29 HIK A 101/76 (2)	Pus, intraoral incision
		24 HIK A 2/76 (2)	Pus, extraoral incision
		25 HIK A 16/76 (2)	Pus, submandibular lesion
		35 HIK *A. israelii*	Source not known
		43 HIK A 495/75	Pus, extraoral incision
		49 HIK A 581/75	Pus, extraoral incision
		42 HIK A 413/75	Pus, perimandibular abscess
		41 HIK A 408/75	Pus, extraoral incision, jaw
		45 HIK A 514/75	Nostril swab (throat abscess)
		15 HIK A 555/75 (2)	Pus, jaw abscess
		28 HIK A 33/76 (2)	Pus, extraoral incision
		Spain 302	Source not known
		Spain 303	Source not known
		285 HIK A 526/76	Pus, extraoral incision, jaw
		A. israelii ATCC 12103 (1)	Cervico-facial actinomycosis
		A. israelii ATCC 12836 (1)	Human actinomycosis
		A. israelii ATCC 13031 (1)	Bovine lumpy jaw
	c	266 HIK A 32/76	Blood and pus, submandibular abscess
		289 HIK A 666/76	Subcutaneous abscess
		294 HIK A 98/77	Granulomatous tissue
		295 HIK A 111/77	Extraoral incision
		301 HIK A 271/77	Pus, jaw abscess
	d	39 HIK A 341/75	Pus, chronic jaw process
		40 HIK A 393/75	Pus, subcutaneous submandibular abscess
		47 HIK A 563/75	Pus, chronic periodontitis
		50 HIK A 589/75	Oral swab
		52 HIK A 662/75	Pus, extraoral incision
		53 HIK A 4/76	Pus, subcutaneous chin abscess
		51 HIK A 634/75	Pus
		55 HIK A 125/76	Pus
		46 HIK A 527/75	Parotid abscess
		38 HIK A 302/75	Pus
		88 HIK A 37/76	Pus, extraoral incision

Table 1 (*continued*)

Cluster	Sub-cluster	Strain name/number*	Origin
Not clustered		44 HIK A 512/75	Pus, jaw cyst
		89 HIK A 43/76	Pus, chin abscess
		A. israelii ATCC 12597 (1)	Human actinomycosis
		345 HIK A 109/79	Pus
		262 HIK A 463/75	Pus, extraoral incision
		268 HIK A 38/76	Pus, jaw lesion (sinusitis)
		LHMC AC4168	Oral isolate, child
2		*Lactobacillus casei* subsp. *rhamnosus* NCDO 243	Source not known
		L. plantarum NCDO 1752	Source not known
Not clustered		54 HIK A 101/76	Pus, intraoral incision
		Actinomyces naeslundii WVU 963 (4)	Human source
3	a	56 HIK A 493/77	Pus, extraoral incision
		A. naeslundii ATCC 12104 (1)	Human sinus after extraction
		58 HIK *A. naeslundii*	Source not known
		59 HIK *A. naeslundii*	Source not known
		69 HIK A 177/76	Sputum
		A. naeslundii WVU 398A (1)	Human dental calculus
		65 HIK A 291/75	Swab, throat phlegmon
		76 HIK 8631/68	Source not known
		77 HIK A 288/77	Intraoral incision
		Poland 20	Human source
		A. naeslundii CDC W752 (2)	Wound abscess
		72 HIK A 433/76	Intraoral incision, jaw
		Poland 12	Human source
		332 HIK *A. naeslundii*	ATCC
		A. naeslundii WVU 398A (1)	Human dental calculus
		86 HIK A 609/75	Pus, perimandibular abscess
		91 HIK A 633/76	Throat abscess
	b	286 HIK A 603/76	Pus, jaw
		LHMC EF269	Oral isolate, child
		LHMC EF1006	Oral isolate, child
		LHMC AM172	Oral isolate, child
	c	*Actinomyces viscosus* ATCC 15987 (1)	Periodontal disease, hamster
		92 HIK *A. viscosus*	ATCC
		A. viscosus WVU 440	Hamster mouth
		333 HIK A 902/74	Human source
		A. viscosus ATCC 19246 (2)	Cervico-facial actinomycosis
		A. viscosus E Be66	Human carious dentine
		A. viscosus E Be32	Human carious dentine
		84 HIK A 360/75	Pus, cheek incision
		A. viscosus ATCC 27044 (WVU 474)	Human sputum
		A. viscosus LHMC B25	Dental plaque, child
		A. viscosus LHMC 8A06	Dental plaque, New Guinea tribesman
		A. viscosus LHMC 11B2	Dental plaque, New Guinea tribesman
		A. naeslundii WVU 820	Human source
		A. naeslundii LHMC B120 (3)	Approximal plaque, child
		A. naeslundii LHMC B102 (3)	Approximal plaque, child
		A. viscosus LHMC B236	Dental plaque, child
		101 HIK A 754/74	Throat swab
		298 HIK A 141/77	Wound abscess
		66i HIK A 292/75	Pus, cheek abscess
		66ii HIK A 292/75	Pus, cheek abscess
		64 HIK A 181/75	Pus, periodontitis, perimandibular
		Poland 22	Human source
		A. viscosus E Be64	Human carious dentine
		LHMC AC2165	Oral isolate, child
		90 HIK A 510/76	Cheek abscess

Table 1 (*continued*)

Cluster	Sub-cluster	Strain name/number*	Origin
Not clustered		LHMC AC3136	Oral isolate, child
		Poland 19	Human source
4		74 HIK A 662/76	Intraoral incision
		263 HIK A 481/75	Pus, gum wound, jaw
		319 HIK A 382/77	Source not known
		347 HIK	Source not known
Not clustered		292 HIK A 27/77	Source not known
		LHMC AC1972	Oral isolate, child
		293 HIK A 67/77	Pus, extraoral incision
		LHMC AM442	Oral isolate, child
		85 HIK A 402/75	Pus, extraoral chronic process
		264 HIK A 509/75	Pus, cheek abscess
		335 HIK	Source not known
		287 HIK A 615/76	Preauricular abscess
		290 HIK A 685/76	Abscess after tooth extraction
		71 HIK A 421/76	Intraoral incision, jaw
5	a	*Bifidobacterium bifidum* NCTC 10471	Stool
		Bifidobacterium bifidum NCTC 10472	Stool
	b	271 HIK A 110/76	Pus, extraoral incision
		272 HIK A 159/76	Pus, extraoral incision
		282 HIK A 457/76	Granuloma, median neck incision
	c	*Eubacterium lentum* ATCC 25559	Rectal tumour
		Eu. alactolyticum ATCC 23263	Purulent pleurisy
6		*Eu. limosum* ATCC 8486	Source not known
		Eu. ventriosum ATCC 27560	Faeces
Not clustered		*Eu. contortum* ATCC 25540	Gangrenous appendicitis
7		*Actinomyces bovis* ATCC 13683 (1)	Bovine lumpy jaw
		A. bovis ATCC 13683 (1)	Bovine lumpy jaw
		95 HIK *A. bovis*	Source not known
8		*Bifidobacterium bifidum* ATCC17930	Source not known
		149 HIK A 595/74	Source not known
9		*Actinomyces bovis* CDC W1755 (2)	Bovine lumpy jaw
		Erysipelothrix rhusiopathiae NCTC 8163	Spleen of pig with endocarditis
		E. rhusiopathiae L C220	Human source
		E. rhusiopathiae L C218	Human source
		E. rhusiopathiae L C219	Human source
		E. rhusiopathiae L C221	Human source
		198 HIK *Corynebacterium pyogenes*	Source not known
10		*Corynebacterium pyogenes* NCTC 6448	Source not known
		C. pyogenes NCTC 6450	Source not known
		C. pyogenes NCTC 5224	Pig
		C. pyogenes NCTC 5225	Pig
		C. pyogenes MAFF C140	Bovine foot infection
		C. pyogenes MAFF C143	Bovine mastitis
		C. pyogenes MAFF C110	Bovine mastitis
		C. pyogenes MAFF P16	Vaginal discharge, pig
		C. pyogenes MAFF P18	Hock joint, piglet
		C. pyogenes MAFF C106	Source not known
11		*Actinomyces odontolyticus* ATCC 17929 (1)	Carious tooth lesion
		A. odontolyticus ATCC 17982 (1)	Carious tooth lesion
		A. odontolyticus CDC W1514 (2)	Dental plaque
		107 HIK A 543/77	Pus, abscess
		320 HIK A 570/77	Pus
Not clustered		*Actinobacterium meyerii* H 8619	Source not known

Table 1 (*continued*)

Cluster	Sub-cluster	Strain name/number*	Origin
12		*Corynebacterium haemolyticum* NCTC 9697	Source not known
		C. haemolyticum NCTC 9998	Source not known
		C. haemolyticum NCTC 8452	Pig
13	a	*Arachnia propionica* ATCC 14157 (1)	Lacrimal canaliculitis
		Ar. propionica 'Fleischmann' (1)	Cervico-facial actinomycosis
		118 HIK A 58/75	Cyst contents, jaw
		119 HIK A 55/76	Pus, submucous abscess
		116 HIK A 313/74	Pus
		Ar. propionica ATCC 29324 (1)	Cervico-facial actinomycosis
	b	110 HIK *Ar. propionica*	Source not known
		Ar. propionica ATCC 29326 (2)	Human actinomycosis
		112 HIK	Source not known
14		194 HIK	Source not known
		300 HIK A 262/77	Intraoral swab
		265 HIK A 570/75	Pus, extraoral incision
		288 HIK A 617/76	Sputum
		278 HIK	Source not known
		267 HIK A 37/76	Pus, extraoral incision
15		*Bacterionema matruchotii* ATCC 14266	Source not known
		B. matruchotii ATCC 14266	Source not known
		128 HIK A 4/76	Pus, subcutaneous abscess
		123 HIK *Bacterionema*	Source not known
		124 HIK A 301/73	Extraoral incision (broken jaw)
		B. matruchotii NCTC 10254	Source not known
		127 HIK A 611/75	Pus
		130 HIK A 72/77	Pus
		129 HIK A 29/76	Sputum
Not clustered		LHMC H341/77	Source not known
		Agromyces ramosus ATCC 25173	Soil
		Actinobacillus sp. ATCC 27072	Vaginal discharge, pig
16	a	*Rothia dentocariosa* ATCC 17931 (1)	Carious teeth, human
		R. dentocariosa v.d.H. B22	Human mouth
		R. dentocariosa CDC W808 (2)	Carious teeth
	b	*R. dentocariosa* v.d.H. B25	Human mouth
		R. dentocariosa v.d.H. B28	Human mouth
		R. dentocariosa v.d.H. B29	Human mouth
		R. dentocariosa ATCC 14189	Carious teeth
17		*Propionibacterium freudenreichii* subsp. *freudenreichii* NCIB 5959	Source not known
		P. freudenreichii subsp. *freudenreichii* NCIB 8896	Source not known
		P. freudenreichii subsp. *shermanii* NCIB 8099	Source not known
18		*P. acnes* ATCC 6919	Facial acne
		P. acnes ATCC 11828	Subcutaneous abscess
19		*P. avidum* ATCC 25577	Source not known
		P. avidum Cu 0589	Source not known
		P. freudenreichii subsp. *shermanii* NCIB 5968	Source not known
20		*P. granulosum* ATCC 25564	Source not known
		P. granulosum C51	Source not known
21		*P. jensenii* NCIB 5960	Cheese
		P. jensenii NCIB 8069	Cheese
		P. lymphophilum ATCC 27520	Submaxillary tissue

Table 1 (*continued*)

Cluster	Sub-cluster	Strain name/number*	Origin
22		*P. thoenii* NCIB 5966	Cheese
		P. thoenii NCIB 8072	Cheese

* Figures in parentheses indicate serotypes.

Sources: ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; CDC, Communicable Disease Center, Atlanta, Georgia, U.S.A.; Cu, Dr C. S. Cummins, Virginia Polytechnic Institute, Blacksburg, Virginia, U.S.A.; E, Dr S. E. Edwardson, University of Lund, Sweden; H, received from Dr K. Holmberg, National Bacteriological Laboratory, Stockholm, Sweden; v.d.H., Dr H. van der Heremer, University of Nijmegen, The Netherlands; HIK, isolated in the Hygiene Institute, University of Cologne, F.R.G.; L, received from Dr D. Jones, University of Leicester, U.K.; LHMC, MRC Dental Epidemiology Unit, London Hospital Medical College, U.K.; MAFF, received from Ministry of Agriculture, Fisheries and Food, Thirsk, Yorkshire, U.K.; NCDO, National Collection of Dairy Organisms, Reading, U.K.; NCIB, National Collection of Industrial Bacteria, Aberdeen, U.K.; NCTC, National Collection of Type Cultures, London, U.K.; Poland, received from Dr P. Heczko, Institute of Microbiology, Medical Academy, Cracow, Poland; Spain, received from Dr A.-C. Velasco, Servicio de Microbiologia, C.S.S.S., Carretera de Andalucia, Madrid, Spain; WVU, West Virginia University, Morgantown, West Virginia, U.S.A.

ensure even distribution of the insoluble substances. The plates were inoculated from suspensions of the bacteria in thioglycollate broth (TB; Becton Dickinson, 11727) and were examined after 21 d incubation.

Hydrogen sulphide production. Slopes of Kligler iron agar (Oxoid, CM33) were stab inoculated from 7 d cultures on BHIA. After incubation for 7 and 14 d, the tubes were examined for blackening of the medium.

Sodium chloride and bile tolerance. Bacteria were inoculated into 2 ml of BHIB plus 2, 4 or 6% (w/v) NaCl and into BHIB with bile salts (Merck, 4054) at a concentration of 5, 10 or 20% (w/v). A control of BHIB only was also included. All inoculations were made from bacterial suspensions in BHIB adjusted to an absorbance at 578 nm (A_{578}) of 1·0. After 7 d incubation, the tubes were examined for growth.

Inhibition by potassium tellurite, sodium azide, sodium selenite, sodium taurocholate and crystal violet. BHIA was used with the inhibitors added from pre-sterilized solutions to give final concentrations of 0·01% (w/v) potassium tellurite, 0·005% (w/v) sodium azide, 0·01% (w/v) sodium selenite, 0·2% (w/v) sodium taurocholate and 0·005% (w/v) crystal violet. It had been established that these concentrations gave rise to differential responses.

Sensitivity to antibiotics. Petri dishes containing a basal layer of 10 ml of iso-sensitest agar (Oxoid, CM471) were overlaid with 9·9 ml of cooled sterile iso-sensitest agar which had been inoculated with 0·1 ml of bacterial suspension. These suspensions were prepared in TB from 4 d cultures and were adjusted to an A_{578} of 1·5. Immediately after solidification of the agar, antibiotic-containing discs (Sensi-Discs; Becton Dickinson) were placed on the agar surface (4 discs per plate; 16 different antibiotics). The plates were checked for the presence or absence of inhibition zones after 2 and 4 d incubation, any sign of inhibition being recorded as positive. Therefore the results cannot be used for clinical purposes.

Susceptibility to lysozyme. Bacteria grown for 7 d on CC agar were suspended in 0·4 ml of sterile distilled water at an A_{578} of 1·0. The suspension was divided into four parts; to two parts were added 0·5 ml of a 1·0% (w/v) solution of lysozyme (Oxoid) in buffer, and to the other two, 0·5 ml of distilled water. The tubes were incubated at 37 °C. After 4 h, one tube containing lysozyme and one control were removed and examined for clearing and for the appearance of a glutinous mass. Both tubes were then shaken and 0·5 ml of 20% (w/v) sodium dodecyl sulphate (SDS) were added; any decrease in turbidity or a total clearing of the suspension was recorded (Mordarska *et al.*, 1978). This was repeated after 24 h on the duplicate set of tubes.

Haemolysis of blood. Haemolysis (α or β) of human, horse, rabbit and sheep blood was tested by incorporating 0·5% (v/v) whole blood into BHIA. Examination was at 7 and 14 d after inoculation.

Production of acid from carbohydrates. The methods used were those described by Schofield & Schaal (1979, 1980*a*).

Aesculin hydrolysis. Commercially prepared 'discs' were used (Becton Dickinson, 25024) and inoculated at the same time as the sugar 'discs', which were used for determination of acid production. A deep brown-black colour in the 'disc' after 4 d incubation indicated a positive result.

Deamination/decarboxylation of amino acids, urease activity, nitrate and nitrite reduction, production of indole. These tests were all performed as described by Schofield & Schaal (1980*b*).

End-products of glucose fermentation. The methods and column packing material were those described by Holdeman & Moore (1972).

API enzyme tests. The commercially prepared kits were from API Systems, Montalieu, Vercieu, France. Initial investigations were carried out using various growth media and preincubation times of the strains and periods of incubation of the strips, to determine the conditions which gave best reproducibility. Bacteria were transferred from BHIA to CC agar and after 7 d incubation were suspended in sterile distilled water to an A_{578} of 1·5; 2 drops from a standard pasteur pipette were placed into each well. The strips were incubated at 37 °C for 6 h before being tested with the reagents provided. The strips were then left exposed to normal daylight for 30 min before the colours were compared with the charts provided. Only those colours showing a colour code of 3 or above were recorded as positive.

All tests were repeated twice.

Coding of the characters. All characters were coded in one of two mutually exclusive states and were scored 1 or 0, giving a total of 124 binary variables for computation, excluding all characters which showed 100% agreement.

Computation. The clustan 1C package of Wishart (1978) was employed. The unweighted pair group average linkage method (Sokal & Michener, 1958) was used in all analyses, single linkage having previously been found to produce 'chaining' effects. The simple matching coefficient (S_{SM}) (Sokal & Michener, 1958) and the similarity coefficient of Jaccard (S_J) (Sneath, 1957) were used together with pattern (D_P) (Sneath, 1968), shape, size and error sum of squares statistics.

RESULTS

At 82·5% similarity (S_{SM}) with average linkage clustering, most strains fell into one of 22 principal clusters. These were defined as containing at least one reference culture and/or more than three strains. Clusters composed of only two or three isolates without reference cultures were not considered in analyses of the results. All but one (cluster 4) of the principal clusters were recovered when using any one of the other coefficients, and variation in their internal structure was slight. As it was not possible to give strain labels in the figures, these are listed in Table 1 in the order in which they occur in the unabridged dendrogram (Fig. 1).

The detailed analysis of the results was mainly based upon the simple matching coefficient, S_{SM} (Fig. 1). For comparison, however, abbreviated dendrograms prepared from both the S_J and D_P analyses are also given (Figs 2 and 3). Since the S_J analysis excludes negative matches, groupings solely due to negative correlations would be uncovered, but the number of strains giving predominantly negative test responses was small and constituted only 0·6% of the total number of organisms. The D_P coefficient reduces the influence of different cell generation rates and sizes (Sneath, 1968). Because members of various genera in several families were included in the study this coefficient might provide valuable additional information. Major discrepancies in the cluster compositions obtained using the three coefficients are discussed below together with the description of the respective clusters. In general, clusters formed at 60% S_J and 90% D_P were equivalent to those at 82·5% S_{SM}.

Cluster 1 contained 59 strains. All except one were previously designated *Actinomyces israelii*; the exception, Cologne isolate HIK68, was originally identified as *Actinomyces naeslundii*. The phenon separated into four subclusters at 84·5% S_{SM} (Fig. 1). Subclusters a and b contained several reference cultures and recent isolates, many previously identified as serotypes 1 or 2. No reference strains were recovered in subclusters c and d, and most of these isolates had not been successfully serotyped in our laboratory. On the edge of this cluster there appeared several strains which are at present uncertainly classified. One such strain was *A. naeslundii* WVU 963, which has been considered to be *A. israelii* (M. A. Gerencser, personal communication) or to be associated with *A. naeslundii* and *A. viscosus* (E. Fillery, personal communication). Cluster composition was the same with the S_J and D_P coefficients but subgroups a and c were amalgamated with the S_J analysis.

Cluster 2 consisted of the only two *Lactobacillus* strains included in the study, *L. casei* subsp. *rhamnosus* and *L. plantarum*, which were linked at 91% S_{SM}.

Cluster 3 was composed of 46 strains mostly designated *A. naeslundii* or *A. viscosus*. At 84·5% S_{SM} it was separated into three subclusters. Subcluster a contained: two strains of *A. naeslundii* serotype 1, WVU 398A being duplicated as one culture was received from the London Hospital Medical College (LHMC) and one directly from WVU; *A. naeslundii*

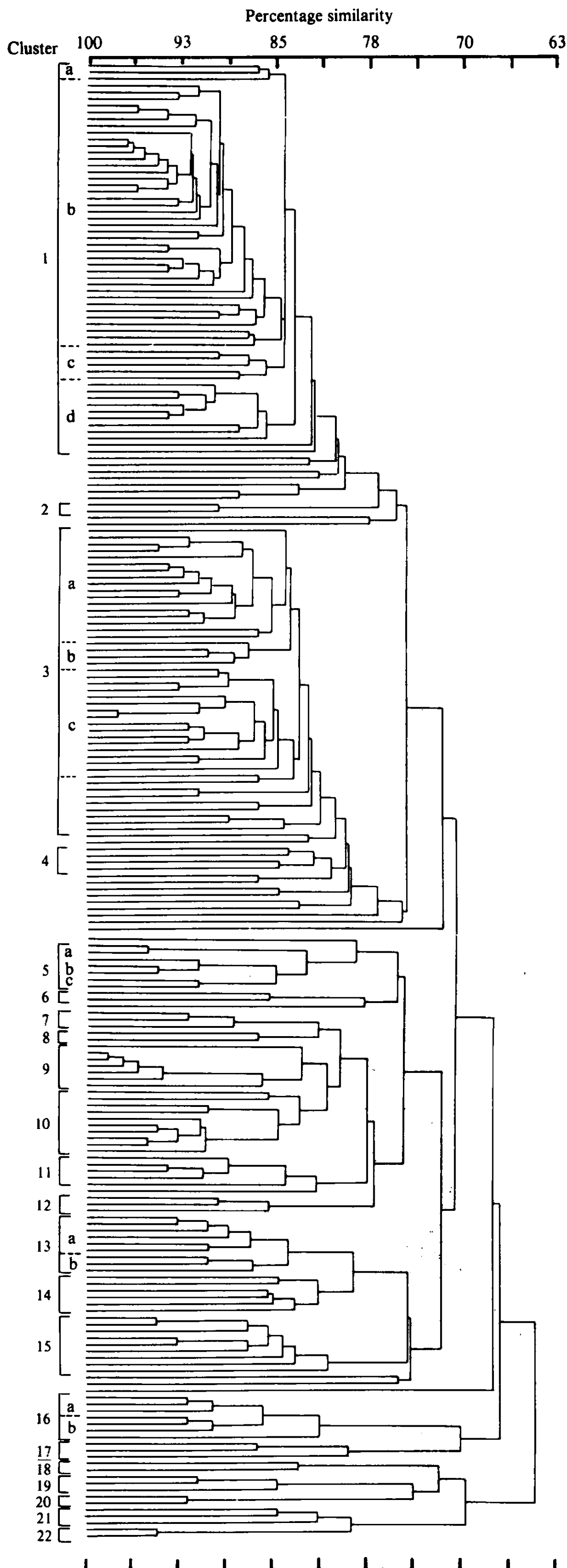

Fig. 1. Dendrogram showing similarity between strains based on the S_{SM} coefficient and average linkage.

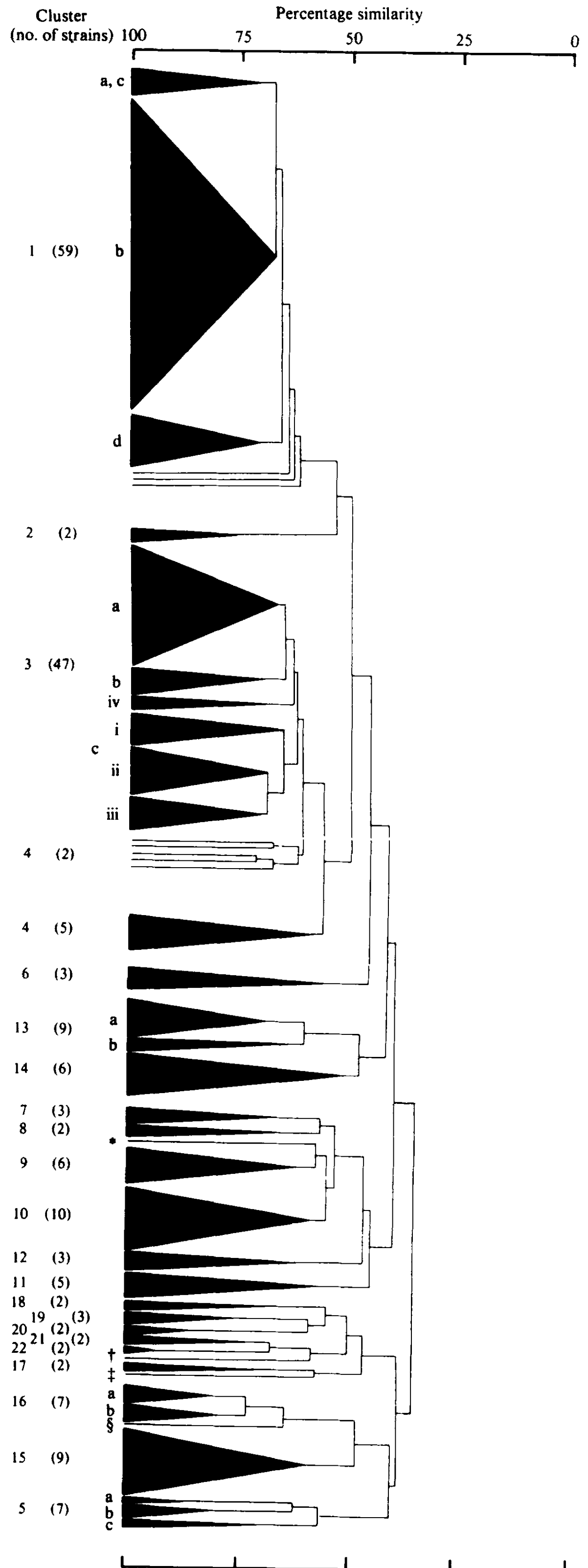

Fig. 2. Abbreviated dendrogram showing similarity between strains based on the S_J coefficient and average linkage.

Single strains: * CDC W1755; † ATCC 27520; ‡ NCIB 8099; § ATCC 14189.

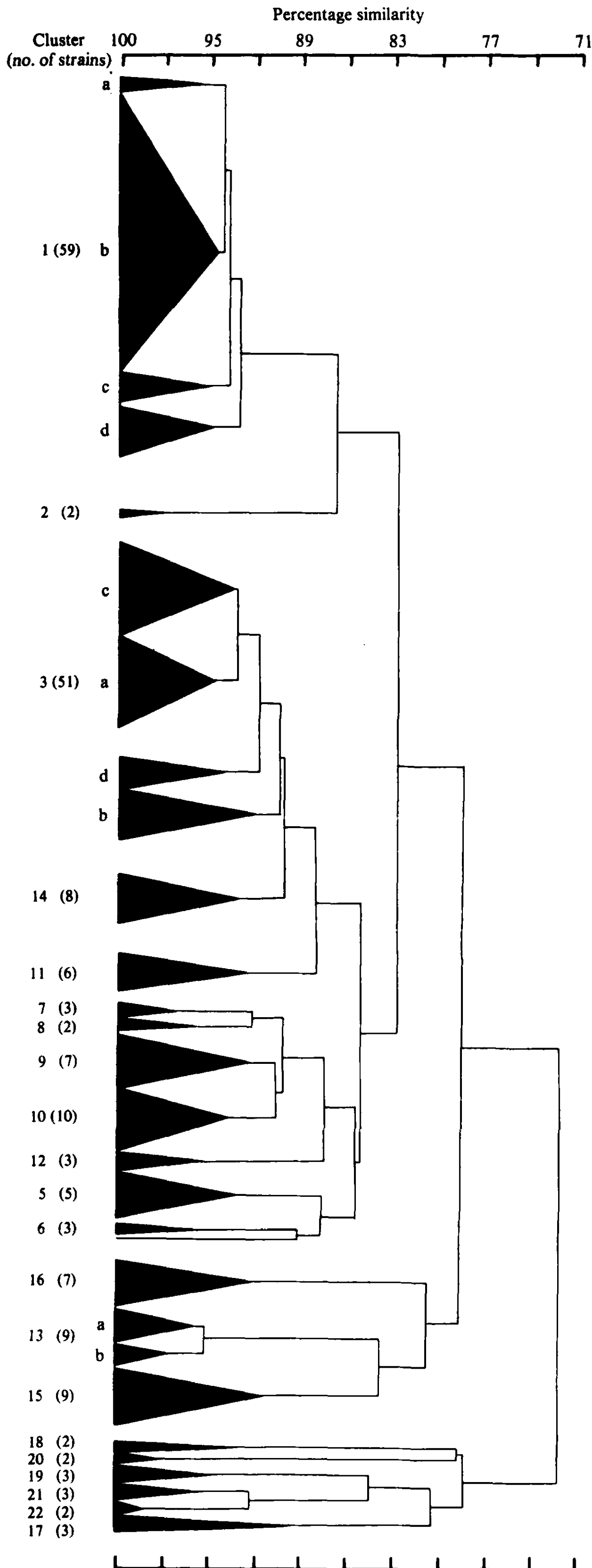

Fig. 3. Abbreviated dendrogram showing similarity between strains based on the pattern coefficient (D_p) and average linkage.

serotype 2 CDC W752; several old reference cultures of *A. naeslundii* from the Cologne collection, and seven recent isolates. At 86% S_{SM}, two isolates labelled *A. viscosus* (HIK86, HIK91) were linked to this *A. naeslundii* subcluster. Subcluster b contained one HIK strain (unidentified) and three oral isolates from young children (LHMC). The majority of subcluster c strains were labelled *A. viscosus* and these fell into one of four smaller subgroups at 88% S_{SM}. One of these small subgroups (i) consisted of *A. viscosus* serotype 1 plus two HIK isolates and WVU 440; this group linked at approximately 85% S_{SM} with the other three subgroups. Subgroup ii contained *A. viscosus* serotype 2, two strains from carious dentine and one HIK strain; subgroup iii consisted of four so-called 'atypical' strains of *A. viscosus* (ATCC 27044, B25, 8A06, 11B2) and *A. naeslundii* WVU 820. Completing subcluster c were the two serotype 3 strains of *A. naeslundii* (B120 and B102) forming subgroup iv, together with an *A. viscosus* strain. The remaining nine strains in cluster 3 were recent isolates of oral origin. Some of these fell into small subgroups at 84·5% S_{SM}, all consisting of only two strains. Alterations to the cluster occurred with the S_J and D_P coefficients. Using S_J, subgroup c (iv) linked directly to subgroup a. With D_P, subgroup b was enlarged with two additional strains from the periphery of cluster 3 and was joined by cluster 4; furthermore, a new subgroup d was formed.

Cluster 4 consisted of only four HIK isolates and occurred on the edge of the *A. naeslundii/A. viscosus* cluster. Although sharing many characters in common with phenon 3, the strains in cluster 4 differed in several respects from *A. naeslundii/A. viscosus* isolates. These included lack of growth under aerobic conditions, more filamentous appearance, hydrolysis of starch, acid production from amygdalin, little or no activity with *meso*-inositol, lactose, melibiose or ribose, tolerance to 4% (w/v) NaCl, absence of lysis by lysozyme after 4 h, and lack of acid phosphatase (Table 2). A relatively large number of isolates, mostly from HIK and LHMC, and which had previously proved difficult to identify, fell on the peripheries of clusters 3 and 4. Using the S_J coefficient, two strains were removed to cluster 3, and two isolates which had joined at 81% to cluster 4 and one unclustered isolate were linked with the two remaining strains of cluster 4.

Clusters 5 and 6. Three distinct subclusters were linked at the relatively low similarity level of 83% in cluster 5. Two *Bifidobacterium bifidum* reference cultures joined at 97% S_{SM} into one subgroup (a), three of the recent HIK isolates formed the second subgroup (b), and two of the five cultures of *Eubacterium* included in the study constituted the third subgroup (c). Two of the remaining three strains of *Eubacterium* formed cluster 6 and were joined at 78% S_{SM} by the third. With the S_J coefficient, cluster 5 moved its relative position and linked at only 39% S_J to clusters 15 and 16. Also at 60% S_J, the subclusters of cluster 5 could be considered as three separate clusters.

Cluster 7 consisted of a duplicated culture of the reference strain of *Actinomyces bovis* ATCC 13683 serotype 1 and one old reference strain from the Cologne collection.

Cluster 8 contained the *Bifidobacterium bifidum* strain ATCC 17930 and one HIK isolate, and was linked to cluster 7 at 81% S_{SM}.

Cluster 9 was composed of one culture of *A. bovis* serotype 2 (CDC W1755), plus a discrete group of all of the strains of *Erysipelothrix rhusiopathiae* included in the study and one recent HIK isolate, which had been tentatively identified as *Corynebacterium pyogenes*. This group linked to the *A. bovis* strain at a relatively low similarity level. The *A. bovis* strain was removed from cluster 9 by the S_J coefficient.

Cluster 10 comprised four reference cultures of *Corynebacterium pyogenes* and six recent isolates all of which were labelled *Corynebacterium pyogenes*.

Cluster 11 contained five strains, three of which were reference cultures of *Actinomyces odontolyticus*, the others being HIK isolates. The single culture of *Actinobacterium meyerii* included in the study linked to this cluster at 82% S_{SM}.

Cluster 12 was formed by a small group of three reference cultures of *Corynebacterium haemolyticum*.

Table 2. *Characteristics of strains contained in major phena as percentages of positive reactions*

Reference cultures associated with phenon	*A. israelii*	*A. naeslundii*/ *A. viscosus*	—	*A. bovis*	*A. odontolyticus*	*Erysipelothrix**	*C. pyogenes*	*C. haemolyticum*	*Arachnia*	*Bacterionema*	*Rothia*
No. of strains	59	46	4	3	5	7	10	3	9	9	7
Cluster no.	1	3	4	7	11	9	10	12	13	15	16
Colony rough	98	18	0	0	20	0	0	0	100	100	100
Colony smooth	2	94	100	100	100	100	100	100	0	0	0
Gram-positive	100	100	100	100	100	0	100	100	100	100	100
Gram-variable	0	0	0	0	0	100	0	0	0	0	0
Cells filamentous	93	15	50	100	20	0	0	0	89	89	29
Cells diphtheroidal	17	94	100	67	100	100	100	100	44	67	86
Aerobic growth	8	74	17	0	20	0	0	0	11	89	100
Enhanced growth with increased CO_2	100	80	100	100	100	100	100	67	100	89	100
Anaerobic growth (absence of CO_2)	29	0	0	0	0	57	20	33	0	0	0
Cytochrome oxidase:											
pale blue	10	74	25	0	0	0	0	0	0	0	29
deep blue	0	3	25	0	0	0	10	0	0	100	71
Catalase	0	33	0	0	0	0	0	0	0	100	100
NO_2^- reduction	2	48	25	0	0	0	0	0	0	0	100
NO_3^- reduction	59	89	75	0	100	0	20	67	100	100	100
DNAase	2	3	25	100	40	14	90	100	0	56	29
Indole	0	0	0	0	0	0	0	0	0	0	0
Hydrolysis tests:											
aesculin	98	61	100	0	20	0	0	0	0	0	86
gelatin	10	2	0	0	0	0	20	0	11	33	43
starch	12	24	75	100	60	0	50	67	11	89	0
casein	1	0	0	0	0	0	90	0	0	11	0
Tween 20	0	0	0	0	0	0	0	0	0	0	0
Tween 40	58	35	25	0	0	0	20	100	0	33	57
Tween 60	59	28	0	0	0	0	10	67	11	57	57
Tween 80	0	0	0	0	20	0	10	0	0	0	0
lecithinase	17	2	0	0	0	0	10	33	0	0	0
lipase – egg yolk	3	2	0	0	0	0	10	33	33	0	0
Alkali produced in peptone-containing media	1	2	0	0	0	14	10	100	0	100	100
Acid from:											
arabinose	54	5	25	0	60	0	20	0	44	11	14
adonitol	3	0	0	0	20	0	10	33	56	0	0
amygdalin	90	7	25	0	0	0	0	0	89	0	0
cellobiose	87	36	50	0	0	0	0	0	0	0	0
dulcitol	2	0	0	0	0	0	0	0	0	0	0
dextrin	97	87	75	67	80	0	60	100	67	0	0
iso-erythritol	2	0	0	0	0	0	0	0	0	0	0
meso-erythritol	8	6	0	0	0	0	0	0	11	0	0
fructose	100	89	100	100	100	100	90	67	100	100	100
galactose	95	95	75	100	20	86	90	67	89	56	14
glucose	100	100	100	100	100	100	100	100	100	100	100
glycerol	8	31	0	100	0	0	0	0	56	0	14
meso-inositol	91	76	0	67	0	0	0	0	22	0	0
lactose	91	80	25	100	60	86	100	100	89	22	14
mannitol	81	4	0	0	0	14	0	0	89	33	0
mannose	93	80	75	0	0	14	50	67	89	67	71
maltose	98	98	75	67	80	14	80	33	89	33	100
melibiose	95	74	25	67	0	0	0	0	11	0	0
melezitose	80	44	0	0	0	0	30	0	11	0	57
raffinose	100	94	50	0	0	0	0	0	100	0	0
rhamnose	36	2	0	0	60	0	0	0	0	0	0

Table 2 (*continued*)

Cluster no.	1	3	4	7	11	9	10	12	13	15	16
ribose	100	52	0	0	0	100	90	23	11	78	0
sorbitol	24	6	0	0	0	0	0	0	78	0	0
sucrose	100	100	100	100	80	14	0	0	90	100	100
trehalose	83	89	25	0	20	0	20	67	56	0	100
xylose	93	14	25	0	40	0	70	0	0	0	14
α-methyl-D-glucoside	32	4	0	0	0	0	0	0	0	0	100
α-methyl-D-mannoside	12	7	0	0	0	14	0	33	0	0	0
Growth in the presence of:											
NaCl 2% (w/v)	47	89	100	33	0	86	50	0	11	67	100
4% (w/v)	7	13	50	0	0	14	0	0	0	11	43
6% (w/v)	2	0	0	0	0	0	0	0	0	0	0
bile 5% (w/v)	29	93	100	33	0	100	80	33	100	11	100
10% (w/v)	15	85	100	33	0	100	80	0	89	0	100
20% (w/v)	2	48	25	67	0	100	60	0	0	0	71
sodium selenite 0·01% (w/v)	10	31	0	33	0	14	0	0	56	78	100
potassium tellurite 0·01% (w/v)	47	61	50	100	20	71	50	0	33	0	100
sodium azide 0·005% (w/v)	91	85	100	33	100	100	100	0	89	100	100
crystal violet 0·005% (w/v)	8	19	25	33	0	86	0	33	11	22	0
sodium taurocholate 0·2% (w/v)	80	48	75	67	0	14	30	67	11	33	100
Growth characteristics on blood agar:											
human – grey/green discoloration	8	14	0	33	20	0	0	0	0	0	0
human – clearing and discoloration	2	7	0	100	60	71	100	100	100	44	86
sheep – grey/green discoloration	2	7	0	0	40	0	20	0	0	0	0
sheep – clearing and discoloration	2	5	0	100	40	14	90	100	0	44	71
horse – clearing and discoloration	0	3	0	67	40	29	100	100	67	11	29
Ammonia from:											
arginine	98	26	0	0	0	100	0	0	11	0	0
lysine	0	2	0	0	0	0	0	0	0	0	14
ornithine	2	0	0	0	0	0	0	0	0	0	29
serine	0	0	0	0	0	0	0	0	56	11	100
alanine	0	0	0	0	0	0	0	0	56	0	0
methionine	0	0	0	0	0	0	0	0	11	0	14
aspartic acid	2	5	0	0	0	0	0	0	22	56	14
glutamic acid	0	0	0	0	0	0	0	0	22	11	0
leucine	0	0	0	0	0	0	0	0	11	0	0
urea	3	50	50	0	0	0	0	0	11	22	0
Decarboxylation of:											
lysine	0	0	0	0	20	0	0	0	0	44	0
ornithine	0	0	0	0	0	0	0	0	44	33	0
methionine	0	0	0	0	0	0	0	0	0	33	0
leucine	0	0	0	0	0	14	0	0	0	0	29
aspartic acid	0	0	0	0	0	14	0	0	0	22	0
glutamine	2	0	0	0	0	0	0	0	0	0	14
Inhibition by (μg per disc):											
nalidixic acid (30)	32	65	75	33	40	86	70	100	11	67	14
sulphamethoxazole trimethoprim (25)	32	89	75	33	80	100	90	100	11	89	100
amikacin (10)	95	100	100	100	80	100	100	100	89	100	86
cefoxitin (30)	100	100	100	100	100	100	100	100	100	100	100
oxytetracycline (30)	98	98	100	100	100	100	100	0	100	100	86
Hyaluronidase	2	2	0	0	20	0	0	0	11	11	72
Chondroitin sulphatase	0	0	0	0	0	14	0	0	0	0	0
Lysis by lysozyme + SDS:											
weak clearing, 4 h	49	28	75	0	0	0	80	0	11	0	0
weak clearing, 24 h	27	0	0	0	0	0	0	0	0	0	0
strong clearing, 4 h	3	63	0	100	100	100	20	0	0	78	100
strong clearing, 24 h	71	100	100	100	100	100	100	100	100	100	100

Table 2 (*continued*)

Cluster no.	1	3	4	7	11	9	10	12	13	15	16
End-products of glucose fermentation:											
acetic acid	100	98	100	100	100	100	100	100	100	100	100
propionic acid	0	0	0	0	0	0	0	0	100	78	14
butyric acid	0	0	0	0	0	0	0	0	0	0	0
isovaleric acid	0	0	0	0	0	0	0	0	0	0	0
caproic acid	0	0	0	0	0	0	0	0	0	0	0
pyruvic acid	0	0	0	0	0	0	0	0	0	44	14
lactic acid	100	98	100	100	100	86	100	100	0	89	100
succinic acid	95	61	25	33	0	100	60	67	0	0	29
API enzyme tests:											
2. alkaline phosphatase	2	0	0	0	0	0	0	33	0	11	0
3. esterase (C4)	64	26	0	0	0	0	10	0	89	100	43
4. esterase lipase (C8)	7	6	0	0	0	0	10	0	100	100	43
5. lipase (C14)	3	2	25	0	0	0	0	0	0	0	0
6. leucine arylamidase	83	93	50	100	100	29	20	0	78	0	100
7. valine arylamidase	1	9	0	0	0	0	0	0	0	0	71
8. cystine arylamidase	0	0	0	0	0	0	0	0	0	0	86
10. chymotrypsin	0	0	0	0	0	71	0	0	0	0	0
11. acid phosphatase	73	31	0	0	0	86	70	100	0	0	14
12. phosphoamidase	64	6	0	0	0	86	0	0	0	11	0
13. α-galactosidase	15	0	0	0	0	14	0	0	0	0	0
14. β-galactosidase	90	57	50	0	0	14	70	100	11	0	0
15. β-glucuronidase	2	5	25	0	0	0	80	0	0	0	0
16. α-glucosidase	97	30	75	0	0	0	0	0	89	78	86
17. β-glucosidase	95	27	25	0	60	0	0	0	0	22	0
18. *N*-acetyl-β-glucosaminidase	2	4	0	100	0	86	90	100	11	11	0
19. α-mannosidase	3	0	0	0	0	0	0	0	0	0	0
20. α-fucosidase	0	0	0	0	60	14	0	0	0	0	0

* Results include *A. bovis* serotype 2 CDC W1755.

Cluster 13 contained only strains of *Arachnia propionica*. At 86% S_{SM} this cluster comprised two subclusters. Subcluster a included ATCC 14157 and ATCC 29324, both serotype 1, the 'Fleischmann' strain and three other HIK isolates. The serotype 2 reference culture and two HIK strains were recovered in subcluster b. Good differential characters for subcluster a were the fermentation of adonitol, *meso*-inositol and trehalose, and the production of ammonia from ornithine, serine and alanine. Of the subcluster b strains, 50% were able to ferment *meso*-erythritol, to produce ammonia from arginine, methionine and urea, and to break down hyaluronic acid, in contrast to the 100% negative response of all subcluster a cultures.

Cluster 14 represented a group of unidentified HIK strains, which linked with cluster 13 at 79% S_{SM}.

Clusters 15 and 16. All strains of *Bacterionema matruchotii* included in the study were recovered in cluster 15. Similarly, cluster 16 consisted only of cultures of *Rothia dentocariosa*, but was divided into two subclusters at 86% S_{SM}.

Clusters 17 to 22 were entirely composed of reference cultures of the genus *Propionibacterium*, the majority of which grouped in accordance with their species designations. With the S_J coefficient, the only change which occurred in these clusters was the separation of *P. lymphophilum* ATCC 27520 from the two strains of *P. jensenii* in cluster 21.

This analysis included only two duplicated reference cultures. However, a previous preliminary computation with 25 duplicated strains without identification labels established the test error as 1·6%. This was calculated by $p/2$, where p is the percentage deviation from 100 (Sneath & Johnson, 1972).

As an aid to identification, the percentage positive reactions of the major taxa are given in Table 2. Identification of members of the *Actinomycetaceae* can be made on many salient

Table 3. *Characters which may be of use in the differentiation of strains of A. naeslundii and A. viscosus (percentages of positive reactions)*

	Cluster 3						
	A. naeslundii subcluster a	Undifferentiated subcluster b	*A. viscosus* subcluster c*				
			i	ii	iii	iv	Total
No. of strains	17	4	4	4	5	2	16
Catalase	0	0	100	100	80	0	81
NO_2^- reduction	88	100	0	0	40	0	19
NO_3^- reduction	100	100	75	75	80	100	81
Hydrolysis of:							
aesculin	94	100	0	0	20	100	19
Tween 40	35	0	0	50	0	0	19
Tween 60	12	0	0	50	40	0	25
Acid from:							
cellobiose	35	75	0	0	40	0	13
glycerol	18	0	0	25	100	50	50
meso-inositol	94	100	0	75	100	100	63
mannose	100	50	50	75	100	100	75
melibiose	100	75	100	100	20	100	69
ribose	35	25	0	100	100	100	69
trehalose	100	75	25	100	100	100	81
xylose	35	0	0	0	0	0	0
Growth in the presence of:							
NaCl 2% (w/v)	77	100	100	100	100	100	100
bile 20% (w/v)	77	25	100	0	40	0	38
sodium selenite 0·01% (w/v)	65	0	0	75	40	50	44
sodium taurocholate 0·2% (w/v)	65	100	50	0	0	0	13
Ammonia from:							
arginine	29	100	0	25	0	0	6
urea	94	75	25	25	0	0	13
Inhibition by nalidixic acid (30 μg per disc)	71	75	100	0	80	100	69
Lysis by lysozyme + SDS:							
weak clearing, 4 h	0	100	50	0	0	50	19
strong clearing, 4 h	100	0	25	75	60	50	56
API enzyme tests:							
3. esterase (C4)	0	50	50	0	20	100	31
17. β-glucosidase	53	0	0	25	0	0	6

* Subgroups: i, *A. viscosus* serotype 1; ii, *A. viscosus* serotype 2; iii, 'atypical' *A. viscosus* strains; iv, *A. naeslundii* serotype 3 strains (B120, B102). The total includes results for the ungrouped strain B236.

features. Aerobic growth is characteristic only of *Rothia*, *Bacterionema* and a few *A. naeslundii* and *A. viscosus* strains. Confusion between *A. viscosus* and *Rothia* can occur, but separation is possible by the cytochrome oxidase test, nitrite reduction, increased pH in peptone-containing media, discoloration of human blood agar and production of ammonia from serine, all of which are usually strongly positive reactions of *Rothia* strains. On the other hand, rothiae do not ferment dextrin, melibiose and raffinose and do not produce ammonia from urea, tests which were positive for *A. viscosus*. Differentiation of *Bacterionema* from *Rothia* can be made by the following characters: hydrolysis of aesculin and starch; production of acid from melezitose, ribose, trehalose and α-methyl-D-glucoside; growth in presence of 10% (w/v) bile and 0·01% (w/v) potassium tellurite; decarboxylation of lysine, ornithine and methionine; production of hyaluronidase, and leucine and cystine arylamidase.

Major problems have existed in the differentiation of isolates of *A. naeslundii* and *A. viscosus*. Suitable diagnostic characters are therefore presented separately in Table 3. The

Table 4. *Characteristics of Propionibacterium strains contained in clusters 17–22 as percentages of positive reactions*

Reference cultures associated with phenon	*P. freudenreichii*	*P. acnes*	*P. avidum/ P. freudenreichii*	*P. granulosum*	*P. jensenii/ P. lymphophilum*	*P. thoenii*
No. of strains	3	2	3	2	3	2
Cluster no.	17	18	19	20	21	22
Colony rough	0	0	0	0	0	0
Colony smooth	100	100	100	100	100	100
Gram-positive	100	100	100	100	100	100
Gram-variable	0	0	0	0	0	0
Cells filamentous	0	0	0	0	0	0
Cells diphtheroidal	100	100	100	100	100	100
Aerobic growth	0	0	0	0	100	100
Enhanced growth with increased CO_2	100	100	100	100	100	100
Anaerobic growth (absence of CO_2)	0	100	67	100	0	0
Cytochrome oxidase:						
pale blue	100	100	100	0	100	100
deep blue	0	0	0	100	0	0
Catalase	100	100	100	100	0	0
NO_2^- reduction	67	0	100	0	33	0
NO_3^- reduction	67	0	100	0	33	0
DNAase	33	0	100	100	0	0
Indole	33	100	0	0	0	0
Hydrolysis tests:						
aesculin	33	0	33	0	33	100
gelatin	0	50	67	50	0	33
starch	0	0	100	100	0	100
casein	0	50	67	0	0	0
Tween 20	0	0	0	0	0	0
Tween 40	33	100	100	100	0	0
Tween 60	0	100	100	100	0	100
Tween 80	0	0	33	0	0	0
lecithinase	0	0	33	100	0	0
lipase – egg yolk	0	0	0	100	0	0
Alkali produced in peptone-containing media	0	0	0	0	0	0
Acid from:						
arabinose	33	0	33	0	67	0
adonitol	67	0	67	0	67	100
amygdalin	0	0	33	0	33	100
cellobiose	0	0	0	0	33	0
dulcitol	0	0	0	0	0	0
dextrin	0	0	100	0	33	100
iso-erythritol	67	50	100	0	100	100
meso-erythritol	33	50	67	0	100	100
fructose	67	100	100	100	100	100
galactose	100	50	100	100	67	100
glucose	100	100	100	100	100	100
glycerol	67	0	33	0	100	100
meso-inositol	67	0	100	0	67	100
lactose	67	0	0	0	67	100
mannitol	0	50	0	0	33	0
mannose	67	100	100	50	100	100
maltose	33	0	100	100	100	100
melibiose	0	0	0	50	33	100
melezitose	0	0	0	50	33	100
raffinose	0	0	0	50	33	0

Table 4 (*continued*)

Cluster no.	17	18	19	20	21	22
rhamnose	0	0	33	0	0	0
ribose	0	50	100	50	100	100
sorbitol	0	50	33	0	33	0
sucrose	0	0	100	100	67	100
trehalose	33	50	67	100	33	100
xylose	0	0	0	0	0	0
α-methyl-D-glucoside	0	0	0	0	0	0
α-methyl-D-mannoside	0	0	0	0	0	0
Growth in the presence of:						
NaCl 2% (w/v)	100	100	100	100	100	100
4% (w/v)	100	50	100	100	33	100
6% (w/v)	100	50	33	100	0	50
bile 5% (w/v)	100	100	100	100	100	100
10% (w/v)	100	100	100	100	100	100
20% (w/v)	67	100	100	100	100	100
sodium selenite 0·01% (w/v)	100	50	33	0	0	50
potassium tellurite 0·01% (w/v)	100	100	67	100	0	0
sodium azide 0·005% (w/v)	100	100	100	100	100	100
crystal violet 0·005% (w/v)	0	0	0	0	33	0
sodium taurocholate 0·2% (w/v)	67	100	67	100	33	100
Growth characteristics on blood agar:						
human – grey/green discoloration	0	0	0	0	0	0
human – clearing and discoloration	67	0	100	100	0	100
sheep – grey/green discoloration	0	0	0	0	0	0
sheep – clearing and discoloration	67	50	100	100	0	100
horse – clearing and discoloration	0	0	100	100	0	100
Ammonia from:						
arginine	33	100	0	0	33	0
lysine	0	0	0	50	0	0
ornithine	0	0	0	0	0	0
serine	100	100	100	100	33	50
alanine	67	0	0	0	0	0
methionine	0	0	0	0	0	0
aspartic acid	100	100	100	50	33	0
glutamic acid	0	0	0	0	0	0
leucine	0	0	0	0	0	0
urea	0	100	100	100	33	0
Decarboxylation of:						
lysine	0	0	0	0	0	0
ornithine	0	0	0	0	0	0
methionine	0	0	0	0	0	0
leucine	0	0	0	0	0	0
aspartic acid	0	0	0	0	0	0
glutamine	0	0	0	0	0	0
Inhibition by (μg per disc):						
nalidixic acid (30)	0	50	0	100	67	0
sulphamethoxazole trimethoprim (25)	33	100	33	100	100	100
amikacin (10)	0	100	0	100	100	100
cefoxitin (30)	33	100	100	100	100	100
oxytetracycline (30)	100	100	0	100	100	100
Hyaluronidase	33	50	100	100	0	0
Chondroitin sulphatase	33	0	100	100	0	0
Lysis by lysozyme + SDS:						
weak clearing, 4 h	0	0	0	0	0	0
weak clearing, 24 h	0	0	0	0	0	0
strong clearing, 4 h	100	50	0	0	0	0
strong clearing, 24 h	100	0	0	0	0	0
End-products of glucose fermentation:						
acetic acid	100	100	100	100	100	100
propionic acid	100	100	100	50	100	100

Table 4 (*continued*)

Cluster no.	17	18	19	20	21	22
butyric acid	0	0	0	0	0	0
isovaleric acid	0	100	67	100	67	100
caproic acid	0	0	0	0	0	0
pyruvic acid	0	0	0	0	0	0
lactic acid	0	0	0	100	0	0
succinic acid	100	50	0	100	33	0
API enzyme tests:						
2. alkaline phosphatase	0	50	0	0	0	0
3. esterase (C4)	67	0	100	100	100	100
4. esterase lipase (C8)	67	50	67	100	100	0
5. lipase (C14)	0	0	0	0	0	0
6. leucine arylamidase	100	0	100	100	100	100
7. valine arylamidase	0	0	0	0	0	0
8. cystine arylamidase	100	0	0	0	67	100
10. chymotrypsin	0	0	0	0	0	0
11. acid phosphatase	0	100	100	0	67	100
12. phosphoamidase	33	100	100	0	67	100
13. α-galactosidase	67	0	0	100	100	0
14. β-galactosidase	33	100	100	0	0	100
15. β-glucuronidase	0	100	0	0	0	0
16. α-glucosidase	100	50	100	100	100	100
17. β-glucosidase	67	0	100	100	100	100
18. *N*-acetyl-β-glucosaminidase	0	100	100	0	0	100
19. α-mannosidase	0	100	100	0	0	100
20. α-fucosidase	0	0	0	0	0	0

most useful are: nitrite reduction; aesculin hydrolysis; production of acid from cellobiose, glycerol and ribose; and production of ammonia from urea. All of these could easily be used for routine identification.

The *A. israelii* phenon exhibits many diagnostic features, particularly the sugar fermentation reactions, lack of growth in presence of bile salts and the production of ammonia from arginine. Tests useful for differentiation of the subclusters are: hydrolysis of gelatin by strains in subcluster a; lack of tolerance to 2% (w/v) NaCl, sensitivity to lysozyme after 4 h and tolerance to potassium tellurite by strains in subcluster b; failure to reduce nitrate by most strains in subcluster c; a mostly diphtheroidal shape and failure to ferment trehalose by subcluster d isolates.

Clusters 7, 9, 10, 11 and 12 are readily distinguished from all other phena. They share many features, but can be separated on the basis of DNAase, starch, casein and Tween hydrolysis, especially clusters 10, 11 and 12. Within these five clusters, the fermentation of glycerol by cluster 7 strains is uncommon, as is the fermentation of ribose by strains in clusters 9 and 10. The production of ammonia from arginine is useful for identification of cluster 9 strains and susceptibility to lysozyme after 4 h is characteristic of cluster 10.

Cluster 13, which contains only strains of *Arachnia propionica*, is characterized by: the pattern of sugar fermentation reactions; production of ammonia from serine and alanine; non-susceptibility to lysozyme after 4 h; production of propionic acid as a major end-product of glucose fermentation; production of esterase and esterase lipase.

Characters useful for the identification of the six clusters of *Propionibacteria* are given in Table 4.

DISCUSSION

As has been noted in many previous studies (Boone & Pine, 1968; de Weese *et al.*, 1968; Holmberg & Nord, 1975; Pine, 1970), *A. israelii* is a well-defined taxon which links at rather

low similarity levels with other species of the genus *Actinomyces*. It is therefore possible that these organisms deserve recognition as a separate genus in the family *Actinomycetaceae*. Further investigations with the serotypes and with strains from the subclusters delimited in cluster 1 may show these subgroups to be equivalent to species in a newly erected genus.

Unfortunately most of the other *Actinomyces* species are not so clearly defined. It has previously been suggested that *A. naeslundii* and *A. viscosus* should be combined (Holmberg & Hallander, 1973; Slack & Gerencser, 1975), a view also supported to a certain extent by several serological studies (Bowden *et al.*, 1976; Collins *et al.*, 1973; Holmberg & Forsum, 1973; Gerencser & Slack, 1967). In the numerical taxonomic study of Holmberg & Nord (1975) five representatives of these species grouped together in one subcluster at 92·5% S_{SM}, the other subcluster consisting of *A. odontolyticus*. In the present study these species once again grouped together (cluster 3), but formed a discrete set of subclusters which were defined by all coefficients employed. One large subcluster contained only *A. naeslundii* strains. The subcluster of *A. viscosus* cultures was divided into four groups. Three represented the animal serotype 1 strains (i), the human serotype 2 strains (ii) and the 'atypical' human *A. viscosus* isolates (iii). These groupings are in good agreement with the recent work of Fillery *et al.* (1978) who used biochemical and cell wall data to separate strains of *A. viscosus* into those of human (serotype 2) and animal (serotype 1) origin and defined a group of 'typical' *A. naeslundii* strains. Using the S_1 nuclease hybridization method with strains from the clusters defined by Fillery *et al.* (1978), Coykendall & Munzenmaier (1979) delineated a group of 'atypical' *A. naeslundii* strains. One of these 'atypical' strains, LHMC B120, was included in our study. It did not fall in the main *A. naeslundii* subclusters and was recovered in subgroup c (iv). Both groups of workers also described an 'atypical' *A. viscosus* strain (W1053 = ATCC 27044 = WVU 474) which in our study clustered with other 'atypical' strains in the small subgroup iii.

There were a relatively large number of ungrouped strains at the peripheries of phena 3 and 4. Many fell into one of these clusters in the D_P analysis, but several single isolates remained outside the groups. Most of these strains were recent isolates and were included in this study because they showed characteristics which differed from those presently used to define *A. viscosus* or *A. naeslundii*.

The species *A. naeslundii* and *A. viscosus* are closely related and possibly should be combined. However, an increasing number of differential characters between these two species are being found, as illustrated here (Table 3) and in the recent work of Fillery *et al.* (1978) and Coykendall & Munzenmaier (1979). *Actinomyces naeslundii* appears to be a more coherent taxon than *A. viscosus*, with the exceptions of serotype 3 strains B120 and B102 which require further investigation. *Actinomyces viscosus* serotypes 1 and 2 are phenotypically different (Table 3) and the genetic evidence (Coykendall & Munzenmaier, 1979) also suggests that they are sufficiently distinct to merit separation, perhaps at the subspecies level. The DNA of the animal strain *A. viscosus* ATCC 15987 serotype 1 contains 60·9 mol % GC whereas that of the human strain ATCC 19246 serotype 2 contains 67·4% (Coykendall *et al.*, 1974). Therefore, at present *A. naeslundii* and *A. viscosus* should remain separate species within which delineation of further smaller subgroups may be possible.

The *Actinomyces odontolyticus* cluster was linked to the *A. naeslundii/A. viscosus* cluster when the pattern coefficient was employed, but with the other coefficients it was joined to the phena containing *A. bovis, C. pyogenes* and *Erysipelothrix rhusiopathiae*. These last three phena were grouped together by all statistical patterns used. The close relationship of *A. bovis* and *C. pyogenes* is particularly interesting. To date, all numerical studies have indicated that *C. pyogenes* and the supposedly closely related species *C. haemolyticum* (Cummins & Harris, 1956; Harrington, 1966) are distinct from other members of the genus *Corynebacterium*, but lack any clear association with other bacterial groups. However, our results do not indicate a very close relationship between *C. pyogenes* and *C. haemolyticum*. Slack & Gerencser (1975) considered the possibility of a relationship between *C. pyogenes, A. bovis* and *A.*

odontolyticus, and our results show that there is probably a close taxonomic association between *A. bovis* and *C. pyogenes.*

Actinomyces bovis, the type species of the genus, was well-separated from the other species of *Actinomyces*. It also possesses a different cell wall composition (Schleifer & Kandler, 1972) having a peptidoglycan type found in many lactobacilli and bifidobacteria. Other species of *Actinomyces* are closely related by cell wall type (Slack & Gerencser, 1975). One reference culture of *Bifidobacterium bifidum* ATCC 17930 (CDC W753) formerly designated *Actinomyces parabifidus*, and one HIK strain previously identified as a *Bifidobacterium* sp. were associated with *A. bovis* at 82% S_{SM}. The other two representatives of the genus *Bifidobacterium* were found in cluster 5.

Erysipelothrix has been associated with the lactic acid bacteria (Sneath & Cowan, 1958; Jones, 1978) and is at present classified as a genus of uncertain affiliation in *Bergey's Manual* (Seeliger, 1974). However, the present study gave little indication of any relationship with *L. casei* or *L. plantarum*. All coefficients used grouped together the *Erysipelothrix* cluster with those of *A. bovis* and *C. pyogenes*. The association may have been based on the large number of negative characters shared by these three species; the clusters joined at 48% with the S_J coefficient.

The three other genera of the family *Actinomycetaceae* – *Rothia, Bacterionema* and *Arachnia* – were grouped together at rather low similarity values (Fig. 1). At present all are single species genera, but other species may exist (Bowden & Hardie, 1978). It has been suggested that serotype 2 *Ar. propionica* strains are sufficiently different from serotype 1 to warrant status as a distinct species (Gerencser & Slack, 1967; Slack & Gerencser, 1975). Our results agree with these proposals, serotypes 1 and 2 being well separated. This is in contrast to the numerical study of Holmberg & Nord (1975) where representatives of serotypes 1 and 2 were linked at 95% S_{SM}. However, these workers included a large number of morphological characters which could have increased the similarity values.

It has been proposed that *Ar. propionica* be moved to the genus *Propionibacterium* (Pine, 1970), but Johnson & Cummins (1972) found low DNA homologies between various species of *Propionibacterium* and *Arachnia* strains. Our results indicate that, at present, the genus *Arachnia* should remain in the *Actinomycetaceae.*

Another genus of uncertain taxonomic affiliation is *Bacterionema*. Strains of this genus formed a distinct cluster having little relationship with other genera of the *Actinomycetaceae* or with any other species studied. The inclusion of *Bacterionema* within the currently defined family *Actinomycetaceae* is doubtful because of the occurrence of mycolic acids, DL-diaminopimelic acid and arabinose in the cell wall. Although our results do not solve this problem, previous work has shown a close relationship with the genus *Corynebacterium* (Minnikin *et al.*, 1978).

In conclusion, our results indicate that the genus *Actinomyces* contains at least three taxa which could merit recognition at the generic level. These are: *A. israelii*; *A. naeslundii, A. viscosus* and *A. odontolyticus*; and *A. bovis*. There is an association between *A. bovis* and *C. pyogenes*, which needs further genetic and chemical investigation. The relationship between *Bifidobacterium bifidum* and *A. bovis* also requires further study. The genus *Arachnia*, which should probably stay within the family *Actinomycetaceae*, could be separated into two species equivalent to serotypes 1 and 2. Of the other genera, *Rothia* may also contain more than one species, and *Bacterionema* may possibly be reclassified in the genus *Corynebacterium.* However, because of the lack of sufficient chemotaxonomic and genetic data it is too soon to propose redefinition of the family *Actinomycetaceae.*

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