Numerical Taxonomy of *Haemophilus*

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A numerical taxonomic study of strains of *Haemophilus*, together with representatives of *Pasteurella* and *Actinobacillus*, showed 12 reasonably distinct phenons. These phenons corresponded in the main with recognized species. Phenon 1 contained *H. influenzae* and *H. aegyptius*, and phenon 2 represented *H. parasuis*. Phenon 3 comprised haemolytic V-requiring strains similar to *H. parahaemolyticus*. Phenons 4 and 5 were very similar, and comprised a number of V-requiring strains including *H. parainfluenzae* and *H. paraphrophilus*. Phenon 6 contained *H. avium* and a few other strains from fowls or swine. Phenon 8 represented *H. paragallinarum* and *H. somnus*, and phenon 9 contained *H. aphrophilus* strains. Phenons 7, 10 and 11 contained strains of *Pasteurella* and *Actinobacillus*, and strains of *H. piscium* were recovered in phenon 12. The last appeared not to belong to the genus *Haemophilus*. A small proportion of strains were unclustered. Although the groupings were broadly satisfactory, they were not as sharply demarcated as phenons in comparable taxonomic studies, and the reasons for this are discussed.

INTRODUCTION

The genus *Haemophilus* was established by Winslow *et al.* (1917) for the influenza bacillus *Haemophilus influenzae* (which was designated as type species) and several other bacteria. These small, Gram-negative rods were all obligate animal parasites, aerobic and rather fastidious in their growth requirements. For growth, *H. influenzae* requires X factor or haematin (Fildes, 1921) and V factor or NAD (Thjotta & Avery, 1921), and the requirement for one or both of these growth factors has been a traditional criterion for inclusion in the genus. The species were separated on growth factor requirements and the animal, host or organ attacked, but recent work (Nicolet, 1968; Sneath & Johnson, 1973) throws doubt on whether species can be based on these attributes. A fuller review is given by Power (1978).

The genus *Haemophilus* lies close to *Actinobacillus* and *Pasteurella*, and the three genera may grade towards the *Enterobacteriaceae*. The genus is not closely associated with the *Brucella*–*Neisseria*–*Moraxella* area (Johnson, 1972; Johnson & Sneath, 1973). A pilot study by Sneath & Johnson (1973) showed three major groups corresponding to the species *H. influenzae*, *H. parainfluenzae* and *H. aphrophilus*. In a more comprehensive study, Kilian (1976*a*) recovered several species including *H. influenzae*, *H. parainfluenzae*, *H. haemolyticus*, *H. haemoglobinophilus*, *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, *H. parasuis*, *H. pleuropneumoniae* and *H. paragallinarum*.

The majority of the named species are listed in Table 1, together with their growth factor requirements and their usual habitats. Since this work was completed, the *Approved Lists of Bacterial Names* have appeared (Skerman *et al.*, 1980), which contain the only scientific names of bacterial taxa that retain their legitimacy, together with the type strains for cultivable species. Although the *Approved Lists* now determine the nomenclature of bacteria, the earlier names and citations, and the earlier proposals for type and neotype strains, have been retained

Table 1. *Some named species of Haemophilus*

Name and citation	Growth factors required	Habitat and comments
<i>H. influenzae</i> (Lehmann & Neumann 1896) Winslow <i>et al.</i> 1917	X, V	Human respiratory tract, type species
<i>H. aegyptius</i> (Trevisan 1889) Pittman & Davis 1950	X, V	Human conjunctivitis
<i>H. haemoglobinophilus</i> (Lehmann & Neumann 1907) Murray 1939	X	Prepuce of dogs
<i>H. parainfluenzae</i> Rivers 1922	V	Human respiratory tract
<i>H. haemolyticus</i> Bergey <i>et al.</i> 1923	X, V	Human respiratory tract; haemolytic, but otherwise similar to <i>H. influenzae</i>
<i>H. parahaemolyticus</i> Pittman 1953	V	Human respiratory tract; similar to <i>H. parainfluenzae</i> but haemolytic
<i>H. gallinarum</i> * Delaplane <i>et al.</i> 1934	X, V	Fowl respiratory tract (contagious coryza); growth factor requirements not well established (de Bleeck, 1931; McGaughey, 1933; Nelson, 1933; Delaplane <i>et al.</i> , 1934)
<i>H. suis</i> * Hauduroy <i>et al.</i> 1937	X, V	Swine respiratory tract; according to Buchanan <i>et al.</i> (1966) the correct name is <i>H. influenzae-suis</i> Lewis & Shope 1931
<i>H. paragallinarum</i> Biberstein & White 1969	V	Fowl respiratory tract
<i>H. parasuis</i> Biberstein & White 1969	V	Swine respiratory tract
<i>H. pleuropneumoniae</i> Shope 1964	V	Swine respiratory tract (porcine contagious pneumonia)
<i>H. influenzae-murium</i> * (Kairies & Schwartz 1936) Lwoff 1939	X	Mouse influenza; requires X only (Ivanovics & Ivanovics, 1937)
<i>H. aphrophilus</i> Khairat 1940	X, 5 % CO ₂	Acute bacterial endocarditis of man; also human respiratory tract
<i>H. paraphrophilus</i> Zinnemann <i>et al.</i> 1968	V, 5 % CO ₂	Human respiratory tract
<i>H. paraphrohaemolyticus</i> Zinnemann <i>et al.</i> 1971	V, 5 % CO ₂	Human respiratory tract
<i>H. piscium</i> Snieszko <i>et al.</i> 1950	Coccarboxylase	Ulcer disease of trout
<i>H. somnus</i> *† Baillie 1969	V	Thromboembolic meningoencephalitis of cattle
<i>H. avium</i> Hinz & Kunjara 1977	V	Fowl
<i>H. segnis</i> Kilian 1977	V	Human mouth (Kilian, 1976 <i>a</i>)
<i>H. ducreyi</i> (Neveu-Lemaire 1921) Bergey <i>et al.</i> 1923	X	Chancroid in man; fastidious to cultivate
<i>H. vaginalis</i> Gardner & Dukes 1955	None	Vaginitis in man

* Species not included in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980).

† Never validly published.

in the present publication because the chequered history of many of the species requires frequent reference to this information. In Table 2, the type strains as now prescribed by the *Approved Lists* are marked TAL; it can be seen that several changes in type strains have been made.

Strains received as *H. ducreyi* were difficult to cultivate and for this reason were not included in the present study. Moreover, no strains were available for the species named *H. citreus* (Diernhofer, 1949), *H. putorum* (Hauduroy *et al.*, 1937), *H. ovis* (Mitchell, 1925) and *H. agni* (Kennedy *et al.*, 1958).

METHODS

Strains. These are listed in Table 2. Strains were checked for purity and stored as suspensions in basal medium broth (see below) containing 10% (v/v) glycerol on beads at -76 °C (Feltham *et al.*, 1978). Working cultures were maintained on chocolate agar. This medium was prepared by melting sterile Oxoid blood agar base, cooling it to 75 °C and adding 6% (v/v) sterile horse blood; the whole was then held at 75 °C and mixed by gentle agitation

Table 2. *Strains studied and their clustering using the S_G coefficient (after making the adjustments noted in the text)*

Phenon	Strain no.	Name as received	Source* and collection no.	Comments†
1	H1	<i>Haemophilus aegyptius</i>	NCTC 8502	TAL. Suggested working type‡; from conjunctiva
	H2	<i>H. aegyptius</i>	NCTC 8134	From conjunctiva
	H3	<i>H. aegyptius</i>	NCTC 8135	From conjunctiva
	H4	<i>H. aegyptius</i>	NCTC 8134	From conjunctiva
	H7	<i>H. influenzae</i>	Kilian HK270	Biotype III; from conjunctiva
	H15	<i>H. haemoglobinophilus</i>	NCTC 1659	TAL. Cotype‡; from prepuce of dog
	H21	<i>H. haemolyticus</i>	NCTC 8479	Suggested working type‡
	H25	<i>H. haemolyticus</i>	WELL CN 5633	
	H26	<i>H. influenzae</i>	NCTC 4560	Suggested working type‡
	H27	<i>H. influenzae</i>	NCTC 8468	Serotype b; from meningitis
	H28	<i>H. influenzae</i>	NCTC 8466	Serotype a; from meningitis
	H29	<i>H. influenzae</i>	ATCC 9006	Serotype a
	H30	<i>H. influenzae</i>	NCTC 8465	Serotype a; from respiratory infection
	H31	<i>H. influenzae</i>	NCTC 7279	Serotype b; from meningitis
	H32	<i>H. influenzae</i>	NCTC 8467	Serotype b; from meningitis
	H33	<i>H. influenzae</i>	NCTC 8469	Serotype c; from sputum
	H34	<i>H. influenzae</i>	NCTC 8470	Serotype d; from throat
	H35	<i>H. influenzae</i>	NCTC 8472	Serotype e
	H36	<i>H. influenzae</i>	NCTC 10479	Serotype e
	H37	<i>H. influenzae</i>	NCTC 7918	Serotype f; from mastoid infection
	H38	<i>H. influenzae</i>	RDHL 9	From oral infection
	H39	<i>H. influenzae</i>	PHLL 27	From sputum
	H40	<i>H. influenzae</i>	RIO 2802	
	H41	<i>H. influenzae</i>	RIO 73357	
	H42	<i>H. influenzae</i>	RIO 72865	
	H43	<i>H. influenzae</i>	PHLL CL17	
	H44	<i>H. influenzae</i>	PHLL 3545	
	H45	<i>H. influenzae</i>	PHLL 43995	
	H46	<i>H. influenzae</i>	PHLL 4532	
	H47	<i>H. influenzae</i>	PHLL 30546	
	H48	<i>H. influenzae</i>	Kilian HK 163	Biotype I; from epiglottis
	H49	<i>H. influenzae</i>	Kilian HK 177	Biotype I; from meningitis
	H50	<i>H. influenzae</i>	Kilian HK 166	Biotype II; from conjunctivitis
	H51	<i>H. influenzae</i>	Kilian HK 219	Biotype II; from sinusitis
	H52	<i>H. influenzae</i>	Kilian HK 209	Biotype IV; from meningitis
	H53	<i>H. influenzae</i>	Kilian HK 343	Biotype IV
	H55	<i>H. influenzae</i>	Kilian HK 233	Biotype V; from otitis media
	H56	<i>H. influenzae</i>	Kilian HK 39	Unclassified strain (Kilian, 1976 a); from pharynx
	H57	<i>H. influenzae</i>	Kilian HK 281	Unclassified strain (Kilian, 1976 a); from conjunctivitis
	H58	<i>H. influenzae</i>	NCTC 8465	Serotype a; from respiratory infection
	H87	<i>H. paraphrohaemolyticus</i>	NCTC 10670	TAL. Holotype (Zinnemann <i>et al.</i> , 1971); from sputum
	H88	<i>H. paraphrohaemolyticus</i>	NCTC 10671	Paratype (Zinnemann <i>et al.</i> , 1971); from sputum
	H128	<i>Haemophilus</i> sp.	PHLL as Johnson J182	From respiratory tract
	H137	<i>Pasteurella ureae</i>	PHLL as Johnson J246	
2	H59	<i>H. suis</i>	NCTC 4557	TAL of <i>H. parasuis</i> . Cotype‡
	H60	<i>H. suis</i>	NCTC 6359	
	H61	<i>H. suis</i>	NCTC 7440	
	H62	<i>H. suis</i>	NCTC 7441	
	H63	<i>H. suis</i>	BU 5479	

Table 2. (*cont.*)

Phenon	Strain no.	Name as received	Source* and collection no.	Comments†
	H64	<i>H. suis</i>	CCM 5747	
	H120	<i>H. parasuis</i>	BU A9	
	H121	<i>H. parasuis</i>	BU C5	
	H122	<i>H. parasuis</i>	BU B26	
	H126	<i>H. parasuis</i>	CCM 5749	
3	H6	<i>H. influenzae</i>	Kilian HK7	Biotype III; from pharynx
	H22	<i>H. haemolyticus</i>	NCTC 10659	TAL. From sputum
	H68	<i>H. parahaemolyticus</i>	BU Shope	Serotype 1
	H69	<i>H. parahaemolyticus</i>	BU K17	
	H70	<i>H. parahaemolyticus</i>	BU Shope	Serotype 1 (Shope, 1964)
	H71	<i>H. parahaemolyticus</i>	BU 1536	Serotype 2
	H72	<i>H. parahaemolyticus</i>	BU 1421	Serotype 3
	H73	<i>H. parahaemolyticus</i>	CCM 5630	
	H74	<i>H. parahaemolyticus</i>	CCM 5869	Serotype 1
	H75	<i>H. parahaemolyticus</i>	CCM 5870	Serotype 2
	H76	<i>H. parahaemolyticus</i>	CCM 5871	Serotype 3
	H78	<i>H. parahaemolyticus</i>	BU 4074	Serotype 1
	H79	<i>H. parahaemolyticus</i>	ATCC 27088	Serotype 1
	H80	<i>H. parahaemolyticus</i>	ATCC 27089	Serotype 2
	H81	<i>H. parahaemolyticus</i>	ATCC 27090	Serotype 3
	H82	<i>H. parahaemolyticus</i>	ATCC 27088	Serotype 1
	H86	<i>H. paraphrophilus</i>	Kilian HK83	From saliva
	H92	<i>H. parainfluenzae</i>	ATCC 7901	
	H118	<i>H. pleuropneumoniae</i>	NCTC 10976	Serotype 2
	H119	<i>H. pleuropneumoniae</i>	Kilian HK361	Serotype 2; from swine pleuropneumonia
4	H24	<i>H. haemolyticus</i>	RDHL 2	From tooth abscess
	H66	<i>H. parahaemolyticus</i>	RDHL 58	From tooth surface
	H67	<i>H. parahaemolyticus</i>	RDHL 17	From tooth abscess
	H83	<i>H. paraphrophilus</i>	NCTC 10557	TAL. Holotype (Zinnemann <i>et al.</i> , 1968)
	H84	<i>H. paraphrophilus</i>	NCTC 10556	Cotype (Zinnemann <i>et al.</i> , 1968); from parietal abscess
	H85	<i>H. paraphrophilus</i>	NCTC 10558	Cotype (Zinnemann <i>et al.</i> , 1968); from trachea
	H89	<i>H. paraphrohaemolyticus</i>	NCTC 10672	Paratype (Zinnemann <i>et al.</i> , 1971)
	H90	<i>H. parainfluenzae</i>	NCTC 7857	TAL
	H93	<i>H. parainfluenzae</i>	ATCC 9796	
	H94	<i>H. parainfluenzae</i>	NCTC 10665	Biotype III; from saliva
	H95	<i>H. parainfluenzae</i>	RDHL 22	From tooth surface
	H98	<i>H. parainfluenzae</i>	BCH 236	
	H100	<i>H. parainfluenzae</i>	Kilian HK47	Biotype I; from pharynx
	H102	<i>H. parainfluenzae</i>	Kilian HK19	Biotype III; from pharynx
	H108	<i>H. gallinarum</i>	LEEDS as ATCC 14385	
	H115	<i>H. segnis</i>	Kilian HK307	From dental plaque
	H116	<i>H. segnis</i>	Kilian HK87	From saliva
5	H91	<i>H. parainfluenzae</i>	NCTC 4101	From tongue
	H96	<i>H. parainfluenzae</i>	NCTC 4101	From tongue
	H104	<i>H. parainfluenzae</i>	Kilian HK408	Unclassified strain (Kilian, 1976 <i>a</i>); NCTC 4101 from tongue
	H105	<i>Haemophilus</i> sp.	Kilian HK164	Taxon A (Kilian, 1976 <i>a</i>); from conjunctivitis
	H106	<i>H. parainfluenzae</i>	ATCC 9796	
	H107	<i>H. gallinarum</i>	NCTC 3438	From fowl respiratory tract
	H112	<i>H. gallinarum</i>	HPRS 2561	From fowl

Table 2. (cont.)

Phenon	Strain no.	Name as received	Source* and collection no.	Comments†
6	H109	<i>H. gallinarum</i>	CCM 5015	Taxon C (Kilian, 1976 a); from fowl
	H110	<i>H. gallinarum</i>	CCM 5066	Taxon C (Kilian, 1976 a); from fowl
	H111	<i>H. gallinarum</i>	CCM 5080	Taxon C (Kilian, 1976 a); from fowl
	H123	<i>H. parasuis</i>	CCM 5111	Taxon C (Kilian, 1976 a); from swine pneumonia
	H124	<i>H. parasuis</i>	CCM 5113	Taxon C (Kilian, 1976 a); from swine pneumonia
	H125	<i>H. parasuis</i>	CCM 5114	From swine pneumonia
	H140	<i>H. avium</i>	Hinz K2654	TAL. Type strain ATCC 29546 (Hinz & Kunjara, 1977); from sinus of fowl
	H141	<i>H. avium</i>	Hinz K2659	From fowl lung
	H142	<i>H. avium</i>	Hinz K94	From sinus of fowl
	H143	<i>H. avium</i>	Hinz K64	From airsac of fowl
7	H131	<i>Actinobacillus lignieresii</i>	Johnson J20 as NCTC 4975	
	H132	<i>Pasteurella multocida</i>	Johnson J167 as NCTC 3195	Suggested working type‡
	H148	<i>Haemophilus influenzae-murium</i>	HCC 95001	From mouse pneumonia
8	H144	<i>H. paragallinarum</i>	Hinz K2403	TAL. From sinus of fowl
	H145	<i>H. paragallinarum</i>	Hinz K2671	From sinus of fowl
	H146	<i>H. paragallinarum</i>	Hinz K1676	From sinus of fowl
	H147	<i>H. paragallinarum</i>	Hinz K1645	From sinus of fowl
	H150	<i>H. somnus</i>	Bailie 3411	From bovine meningitis
	H151	<i>H. somnus</i>	Bailie 2260	From bovine meningitis
	H152	<i>H. somnus</i>	MAFF 91	From bovine meningitis
	H153	<i>H. somnus</i>	MAFF 92	From bovine meningitis
	H154	<i>H. somnus</i>	Bailie 5075	From bovine meningitis
	H155	<i>H. somnus</i>	Bailie 3409	From bovine meningitis
9	H156	<i>H. somnus</i>	Bailie 2830	From bovine meningitis
	H157	<i>H. somnus</i>	Bailie 4060	From bovine meningitis
	H8	<i>H. influenzae</i>	Kilian HK294	Biotype III; from conjunctivitis
	H9	<i>H. aphrophilus</i>	NCTC 5908	TAL. From endocarditis
	H10	<i>H. aphrophilus</i>	NCTC 5886	Holotype‡; from endocarditis
	H11	<i>H. aphrophilus</i>	CIP 5961	
	H12	<i>H. aphrophilus</i>	CIP 6473	
10	H13	<i>H. aphrophilus</i>	CIP 6811	
	H133	<i>Pasteurella pneumotropica</i>	Johnson J169 as NCTC 8141	TAL
	H134	<i>P. haemolytica</i> A	Johnson J171 as NCTC 10365	
	H135	<i>P. haemolytica</i> var. <i>ureae</i>	NCTC 10219	TAL of <i>P. ureae</i>
	H136	<i>P. ureae</i>	Johnson J170 as NCTC 10222	
11	H130	<i>Actinobacillus lignieresii</i>	Johnson J20 as NCTC 4975	
	H139	<i>A. equuli</i>	Johnson J19 as NCTC 8529	TAL. Suggested working type‡
12	H159	<i>Haemophilus piscium</i>	ATCC 15711	Authentic strain (Snieszko <i>et al.</i> , 1950); from trout ulcer
	H160	<i>H. piscium</i>	ATCC 14362	Authentic strain (Snieszko <i>et al.</i> , 1950); from trout ulcer
	H161	Fish isolate	Weymouth 145	From trout

Table 2. (cont.)

Phenon	Strain no.	Name as received	Source* and collection no.	Comments†
	H162	Fish isolate	Weymouth 463	
	H163	Fish isolate	Weymouth 469	
	H164	Fish isolate	Weymouth A	
Ungrouped	H5	<i>Haemophilus influenzae</i>	Kilian HK71	Biotype III; from pharynx
	H14	<i>H. aphrophilus</i>	CIP 6534	
	H16	<i>H. haemoglobinophilus</i>	Johnson J79	
	H17	<i>H. haemoglobinophilus</i>	NCTC 8540	
	H18	<i>H. haemoglobinophilus</i>	NCTC 10619	
	H19	<i>H. haemoglobinophilus</i>	RDHL 35	From dog
	H20	<i>H. haemoglobinophilus</i>	Kilian HK365	
	H23	<i>H. haemolyticus</i>	LEEDS Ryan II	Biotype V; from otitis media TAL. Cotype‡; from vagina Isolated from porcine pneumonia by T. W. A. Little
	H54	<i>H. influenzae</i>	Kilian HK223	
	H65	<i>H. vaginalis</i>	NCTC 10287	
	H77	<i>H. parahaemolyticus</i>	BU 349	
	H97	<i>H. parainfluenzae</i>	RDHL 41	From tooth surface
	H99	<i>H. parainfluenzae</i>	LEEDS MB	
	H101	<i>H. parainfluenzae</i>	Kilian HK14	Biotype II; from pharynx Unclassified strain (Kilian, 1976 a); from saliva
	H103	<i>H. parainfluenzae</i>	Kilian HK80	
	H113	<i>H. paragallinarum</i>	Kilian HK364	TAL. Type strain (Kilian, 1976 a); from dental plaque
	H114	<i>H. paragallinarum</i>	NCTC 10926	
	H117	<i>H. segnis</i>	NCTC 10977	
	H127	<i>Eikenella</i> sp.	RDHL 18	From dental abscess From perianal abscess (Ryan, 1968)
	H129	<i>Haemophilus</i> sp.	NCTC 10555	
	H138	<i>Pseudomonas pertucinogena</i>	Feltham	Type strain (Kawai & Yabuuchi, 1975)
	H149	<i>Haemophilus haemolyticus</i>	ATCC 10014	TAL
	H158	Carp isolate	Bootsma V75/93	From carp erythrodermatitis, possibly a variant of <i>Aeromonas salmonicida</i>

* Sources: ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; Bailie, W. E. Bailie, College of Veterinary Medicine, Manhattan, Kansas, U.S.A.; BCH, R. H. George, Department of Microbiology, The Children's Hospital, Birmingham, U.K.; Bootsma, R. Bootsma, Vakgroep Pathologie, Utrecht, Netherlands; BU, J. Nicolet, University of Berne, Switzerland; CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia; CIP, Collection de l'Institut Pasteur, Paris, France; Feltham, R. K. A. Feltham, Department of Microbiology, University of Leicester, U.K.; HCC, Hungarian National Collection of Medical Bacteria, Budapest, Hungary; Hinz, K.-H. Hinz, Institut für Geflügelkrankheiten, Hanover, Germany; HPRS, E. G. Harry, Houghton Poultry Research Station, Cambridge, U.K.; Johnson, R. Johnson, Department of Microbiology, University of Leicester, U.K.; Kilian, M. Kilian, Department of Microbiology, Royal Dental College, Aarhus, Denmark; LEEDS, J. Frazer, Department of Microbiology, University of Leeds Medical School, U.K.; MAFF, Ministry of Agriculture, Fisheries and Food, Weybridge, Surrey, U.K.; NCTC, National Collection of Type Cultures, Colindale, London, U.K.; PHLL, Public Health Laboratory, Leicester, U.K.; RDHL, W. Sims, Royal Dental Hospital, London, U.K.; RIO, D. C. Turk, Bacteriology Department, Radcliffe Infirmary, Oxford, U.K.; WELL, Wellcome Research Laboratories, Beckenham, Kent, U.K.; Weymouth, A. W. Salisbury, M.A.F.F., Weymouth, Dorset, U.K.

† TAL indicates a type strain in the *Approved Lists of Bacterial Names* (Skerman *et al.*, 1980). Serotypes a-f are those of Pittman (1931). Serotypes 1-3 are those of Nicolet (1971) and Kilian *et al.* (1978). The biotypes are those of Kilian (1976 a).

‡ Sneath & Skerman (1966).

for about 10 min or until the blood became chocolate brown. Strain H158 required a specialized maintenance medium (R. Bootsma, personal communication) consisting of Oxoid tryptose blood agar base with 10% (v/v) sterile horse serum.

Basal medium. The colourless basal medium (BM) contained: Oxoid nutrient broth no. 2, 920 ml; NAD (250 mg l⁻¹ aqueous solution), 10 ml; haematin (250 mg l⁻¹ solution in 10 mM-NaOH), 10 ml; cocarboxylase

(200 mg l⁻¹ aqueous solution), 10 ml; Difco sterile horse serum, 50 ml. Solutions were sterilized by membrane filtration (220 nm pore size). BM agar was prepared by adding 1.5% (w/v) Oxoid agar no. 3.

Standard cultural conditions. Except where stated, stock cultures and tests were incubated at 37 °C in 5–10% (v/v) CO₂ in air. Strains H158 to H164 were incubated at 25 °C in air. Initially, the CO₂ was obtained using Beogex Suppositories (Pharmax, Bexley, Kent) and disposable CO₂ generator envelopes (Becton, Dickinson Co., Wembley, Middx.); later, a CO₂ incubator (Continuous Flow Type GFC) was used. The standard inoculum was one loopful of 24 h culture on maintenance medium suspended in BM to give the same turbidity as tube no. 2 on the MacFarland scale. When media were supplemented with X factor, V factor and cocarboxylase, the final concentrations were, respectively, 2.5, 2.5 and 2.0 mg l⁻¹.

Colonial and cell morphology. Strains grown on chocolate agar were examined for colonial features after 24 h (48 h for more slowly growing strains). Heat-fixed films of 24 h cultures were stained by Burke's modification of Gram's stain (Cruikshank *et al.*, 1975) with 2% (w/v) aqueous safranin as the counterstain.

Growth at different temperatures. Strains were incubated on chocolate agar at 4, 25, 30, 37 and 45 °C and growth was recorded at 7 d for 4 °C and 3 d for the other temperatures.

Growth in different atmospheres. Chocolate agar plates were inoculated and incubated in air, in 5–10% CO₂ in air, and anaerobically. Growth was recorded after 48 h.

Growth on routine media. Nutrient agar (Oxoid) and MacConkey agar (Oxoid) plates were inoculated with a loopful of 24 h culture in BM medium, and examined after 48 h. Growth on horse blood agar was determined after 48 h incubation on BM agar containing 10% (v/v) sterile horse blood in the top layer. Haemolysis of horse, sheep and rabbit blood on layered BM agar was recorded at 48 h. Although β -haemolysis was readily detected, diffuse and incomplete haemolysis (usually of a greenish brown tinge) was seen with many strains – this is referred to as 'greening'.

Growth factor requirements. Cultures were plated on BM agar and BM agar supplemented with haematin (2.5 mg l⁻¹), NAD (2.5 mg l⁻¹), haematin and NAD (both 2.5 mg l⁻¹) or cocarboxylase (2.0 mg l⁻¹); growth was recorded after 48 h.

The presence of enzymes for converting added δ -aminolaevulinic acid to porphyrins (δ -ALA test) was examined by the method of Kilian (1974); a positive result was a pink coloration in the test solution (indicating the presence of porphyrins); a negative result indicated X-dependence.

Motility. This was examined by the method of Craigie (1931) using semi-solid BM containing 0.25% (w/v) Oxoid agar no. 3, and looking for spreading growth after 48 h.

Biochemical tests. Urease production was determined after 48 h, using a modification of Christensen's medium (Cowan, 1974) with added X, V and cocarboxylase. Oxidase activity was tested by the method of Kovacs (1956) with 24 h cultures on chocolate agar. Catalase activity was tested by adding H₂O₂ (20 vol.) dropwise to 24 h plate cultures on BM agar, and noting effervescence. Nitrate reduction in BM broth cultures to which 0.1% (w/v) KNO₃ had been added was tested after 5 d incubation by the method of Wilson & Miles (1964); negative results were checked by adding Zn dust. Activity in the ONPG test was determined after 24 h in liquid medium (Cowan, 1974) with added X, V and cocarboxylase.

For the methyl red and Voges–Proskauer tests, the medium contained 0.5% (w/v) Oxoid peptone, 0.5% (w/v) KH₂PO₄, 0.5% (w/v) glucose and the standard amounts of X, V and cocarboxylase. Cultures were tested after 72 h by adding methyl red. Acetoin production (the Voges–Proskauer reaction) was determined in the same tubes by adding 0.5 ml 1% (w/v) α -naphthol and 1 ml 40% (w/v) NaOH: a positive reaction was indicated by a deep red coloration after 4 h.

Indole production was tested in BM broth cultures after 48 h by adding 0.5 ml Kovacs' indole reagent (Cowan, 1974). Hydrogen sulphide production from peptone was tested in BM cultures after 48 h with lead acetate indicator paper. Gelatin hydrolysis was tested after 72 h on BM agar with 4 g gelatin l⁻¹ by flooding the plates with the reagent of Frazier (1926). Tyrosine and xanthine hydrolysis were determined by the methods of Gordon & Smith (1955). Aesculin hydrolysis was detected by a dark coloration after 48 h on BM agar containing 0.05% (w/v) ferric ammonium citrate and 0.5% (w/v) aesculin. Casein hydrolysis was detected on BM agar with 20% (w/v) sterile skim milk (Oxoid) by examining for zones of clearing for up to 14 d. Hydrogen cyanide production was determined on BM agar with an extra 5% (v/v) sterile horse serum by the method of Sneath (1956), using picric acid/sodium carbonate indicator papers (Sneath, 1966). Deoxyribonuclease activity was tested after 48 h by the method of Smith *et al.* (1969) using DNAase agar (Difco) with added growth factors.

The utilization of mucate, gluconate, pectate, citrate, malonate and acetate was determined in API 50E galleries (API, Farnborough, Hants.) and read after 48 h according to the manufacturer's instructions. To examine the oxidative–fermentative utilization of glucose, the medium of Hugh & Leifson (1953) with added X, V and cocarboxylase was used and tests were read after 72 h.

Other enzyme activities were investigated using the API-ZYM system. Overnight cultures on chocolate agar were suspended in sterile distilled water to give a turbidity between that of tubes no. 5 and no. 6 on the MacFarland scale, and added to the cupules. After 4 h at 37 °C the test reagent, made up according to the manufacturer's

instructions, was added. The plates were left for 10 min under intense light, and then read and scored according to the API standard colour chart. The enzyme activities are recorded in Table 4, tests 40–58. Lipase and tetrathionate reductase (tests 135, 136) were determined in API 50E galleries after 48 h according to the manufacturer's instructions.

Production of acid from carbohydrates. API 50E galleries were used for 39 carbohydrate fermentations (Table 4, tests 70–106, and dulcitol and amylose). Growth from overnight chocolate agar cultures was emulsified in Difco nutrient broth with added factors to give a suspension with the same turbidity as tube no. 2 on the MacFarland scale. The cupules were inoculated with bacterial suspension and the galleries were read at 3, 6, 24 and 48 h. The results obtained by this method were compared with those obtained with Replidishes (Sterilin, Teddington, Middx.), inoculated as described by Stevens (1969), in the following medium (modified from Hugh & Leifson, 1953): Oxoid Lab Lemco, 1 g; Oxoid peptone, 1 g; NaCl, 1 g; KH_2PO_4 , 0.3 g; Oxoid agar no. 3, 5 g; bromothymol blue (0.2%, w/v, aqueous solution), 15 ml; distilled water, 820 ml, to which was added horse serum, 50 ml; carbohydrate (10%, w/v, aqueous solution), 100 ml; and X, V and cocarboxylase. The results were read after 1, 2, 3, 5 and 7 d. An alkaline reaction in the medium containing dulcitol (which was not acidified by any strain) was also recorded if it occurred (test 107).

Tolerance to heat and chemical inhibitors. Growth was observed on BM agar containing 0, 0.5, 1.0, 3.0, 5.0 and 10% (w/v) NaCl after 48 h. In addition, the stimulatory effect of 0.5% NaCl was recorded for cultures incubated in air. To examine resistance to heat, overnight BM cultures were heated to 56 °C, and samples were removed after 0, 5, 10, 15 and 20 min to chocolate agar which was examined for growth after 24 h. Growth on chocolate agar containing potassium tellurite (320 mg l⁻¹) or sodium azide (72 mg l⁻¹) was examined after 48 h. Growth on BM agar containing 0.1% (w/v) phenol was examined after 48 h.

Antibiotic sensitivity. Sensitivity to antibiotics was examined on chocolate agar plates poured to a depth of 5 mm. A loopful of growth from a 24 h agar culture was emulsified in 2.5 ml sterile saline and poured on to each chocolate agar plate. The excess liquid was removed with a sterile Pasteur pipette and then the plates allowed to stand for 30 min before the antibiotic discs were placed aseptically on to the surface. The plates were dried and incubated at 37 °C for 48 h. The zone of growth inhibition was measured. The antibiotics (Multodisks, Oxoid) and quantity per disc were: chloramphenicol, 10 and 50 µg; chlortetracycline, 10 µg; furazolidone, 50 µg; neomycin, 10 µg; oxytetracycline, 10 µg, penicillin G, 1.5 and 5 units; streptomycin, 10 and 25 µg; sulphafurazole, 100 and 500 µg; ampicillin, 25 µg; cephaloridine, 25 µg; carbenicillin, 100 µg; colistin sulphate, 10 µg; gentamicin, 10 µg; co-trimoxazole, 25 µg; tetracycline, 50 µg; erythromycin, 50 µg; novobiocin, 30 µg; oleandomycin, 10 µg.

Numerical taxonomy. The computing was performed on the University of Nottingham's ICL 1900 computer and the University of Leicester's CDC CYBER 72 computer, using programs written by M. J. Sackin and associates of the Microbiology Department, Leicester University.

The $t \times n$ matrix was composed of 164 strains and 141 characters. Two-state characters were scored 0 for negative and 1 for positive, Multi-state characters were scored 0 for negative and 1, 2 for weak and strong positive, respectively. Sensitivity to antibiotics was scored according to the radius of the zone of inhibition around the disc: <1 mm = 0, 1–4 mm = 1, 4–8 mm = 2, >8 mm = 3. The API-ZYM enzyme tests were scored according to the manufacturer's scale of 0–5. The API 50E tests were scored after 48 h incubation and the numbers on the manufacturer's scale of 0–5 were then converted to scores for numerical taxonomy: 0–1 = 0, 2–3 = 1, 4–5 = 2. To give the percentages in Table 4, scores of 0 were taken as negative and 1 or more as positive, except for the API-ZYM and API 50E tests, for which 0–1 was scored as negative and 2–5 as positive.

The similarity coefficients used were the Gower coefficient (S_G) and the pattern coefficient (S_P) with cluster analysis by average linkage (UPGMA) and single linkage. The vigour coefficient of each strain was also calculated. Details of these methods are given in Sneath & Sokal (1973). The scores were all converted to positive and negative for the pattern and vigour coefficients and for the S_{SM} values in Table 3, by using the scoring in Table 4. Test reproducibility was taken into consideration by duplicating six strains. The pairs were: H2, H4; H30, H58; H68, H70; H91, H96; H93, H106; H130, H131.

RESULTS

Numerical taxonomy

Test reproducibility. The results for the six duplicated strains showed that carbohydrate fermentations in Replidishes were somewhat less reproducible than those in API 50E kits. For this reason the Replidish results were omitted from the computations, leaving 141 characters. The resulting similarities between the duplicate strains ranged from 84.0 to 97.1% (average 89.2%) for S_G and from 85.0 to 98.0% (average 90.3%) for S_P . This corresponds to about 5% test error (or rather less if differences in quantitative characters are discounted) which is acceptable (Sneath & Johnson, 1972).

Fig. 1. Dendrogram of 164 strains of *Haemophilus* and related bacteria by UPGMA with Gower's coefficient (S_G). The cophenetic correlation was 0.82.

Fig. 2. Simplified dendrogram from Fig. 1 based on S_G and UPGMA clustering. The numbers of strains in the phenons are those after making the adjustments noted in the text.

Cluster analysis. The results of the different computations gave broadly similar groupings. The taxonomy described below is based on the well-tried technique of UPGMA clustering on the Gower coefficient (Figs 1, 2), with reference to the dendrogram (Fig. 3) from the less-used pattern coefficient as required. One hundred and forty of the strains fell into 12 clusters (Fig. 1). Salient relations are shown in the simplified dendrogram (Fig. 2). Single linkage clustering gave similar but less clear-cut results than UPGMA.

After considering the results from the UPGMA analysis on the pattern coefficient (Fig. 3; cophenetic correlation 0.78), a small number of strains were moved to other phenons. These strains are mentioned below in the descriptions of the phenons and are shown in their

S_p/UPGMA positions in Table 2. For the amended phenons the mean intergroup and intragroup similarities, and mean vigour coefficients, were calculated (Table 3), and also the percentages of positive test results shown in Table 4.

Table 3. *Mean intergroup and intragroup similarities (S_{SM}) and vigour of the phenons*

This table is based on the form of data in which all the scores have been converted to positive and negative (see Methods: *Numerical taxonomy*). The diagonal entries in the table are the mean percentage S_{SM} similarities of all pairs of strains within the corresponding phenon; the off-diagonal entries are the mean similarities between all pairs of strains, one in each of the two corresponding phenons. The mean vigour of each phenon is the mean proportion of characters that have positive state, averaging over all the strains in the corresponding phenon; s.d. indicates the standard deviations of these proportions.

Phenon and name	1	2	3	4	5	6	7	8	9	10	11	12
1. 'influenzae'	83.5											
2. 'parasuis'	78.7	82.5										
3. 'parahaemolyticus'	78.6	77.9	84.1									
4. 'mixed para'	78.4	78.7	80.2	83.6								
5. 'parainfluenzae'	75.3	76.9	78.4	80.4	83.4							
6. 'avium'	75.2	74.3	76.2	78.0	77.7	84.0						
7. 'Actinobacillus-Pasteurella'	75.9	77.0	77.9	77.7	77.7	76.6	80.2					
8. 'somnus'	76.7	76.8	75.9	75.6	71.8	70.3	74.0	84.6				
9. 'aphrophilus'	74.7	76.4	78.1	79.2	77.6	74.5	76.0	74.6				
10. 'Pasteurella'	73.3	75.6	74.7	75.8	77.0	73.5	78.4	70.6	82.2			
11. 'Actinobacillus'	72.4	72.8	78.5	74.9	75.7	71.4	75.6	68.8	76.3	86.2		
12. 'piscium'	60.3	61.3	62.7	63.8	66.9	64.8	68.0	62.0	67.1	65.8	63.5	81.8
No. of strains	44	10	20	17	7	10	3	12	6	4	2	6
Vigour: Mean	0.430	0.439	0.457	0.413	0.464	0.464	0.464	0.368	0.448	0.458	0.551	0.514
S.D.	0.037	0.038	0.030	0.039	0.033	0.038	0.053	0.043	0.044	0.022	0.026	0.068

Description of the phenons

Phenon 1 (the 'influenzae' cluster) contained most of the strains that required both X and V. The majority were non-haemolytic, but about a third of the strains showed some degree of green discoloration on blood agar plates. The inner core of this phenon was composed almost entirely of *H. influenzae* strains including representatives of the Pittman serotypes and Kilian biotypes I, II, IV and V. The strains received as *H. aegyptius* formed a tight subgroup at the 90% similarity level. Strain H137 clustered with this subgroup but its properties suggest that it has become mislabelled since the work of Sneath & Johnson (1973). Phenon 1 also contained the type strains of *H. haemoglobinophilus* (H15) and *H. paraphrohaemolyticus* (H87). A group of eight atypical strains joined the phenon at a similarity of 80 to 85%. When the pattern coefficient dendrogram was considered some strains appeared to be misplaced in the S_G dendrogram. The type strain of *H. segnis* (H117) became a satellite of phenon 8a but did not group well with any other cluster, so it was transferred to the ungrouped strains. Strains H22 (*H. haemolyticus*) and H67 (*H. parahaemolyticus*) moved into phenons 3 and 4, respectively, and were transferred to those groups. A strain of *H. influenzae* (H57) moved from phenon 3 into phenon 1 on the pattern coefficient computation, and as it required both X and V it seemed better placed there.

Phenon 2 (the 'parasuis' cluster) contained strains which were from swine but required only V factor. Six strains received as *H. suis* had lost their X dependence (if they ever possessed it). One strain labelled *H. parasuis* (H122) moved to phenon 2 from the ungrouped strains in the pattern coefficient dendrogram, and was transferred to this phenon.

Phenon 3 (the 'parahaemolyticus' cluster) contained mainly haemolytic V-requiring strains, predominantly porcine in origin. All three serotypes were included. Duplicates of the Shope strain (H68 and H70) were 93% similar. Two strains of *H. pleuropneumoniae* (H118 and H119) were also in phenon 3. One strain of *H. paraphrophilus* (H86) appeared here and not with the other three strains in phenon 4: it differed from the latter in being oxidase positive, but this alone would not account for its position. Some non-haemolytic strains showed greening.

Phenon 4 (the 'mixed para' group) contained V-requiring strains from various sources. It contained seven strains labelled *H. parainfluenzae* (including the type strain H90), the holotype and two cotypes of *H. paraphrophilus*, and one strain of *H. paraphrohaemolyticus* (H89). There were strains of several other species in the phenon, and possibly some are mislabelled. In the pattern coefficient dendrogram *H. segnis* H116 (phenon 8 in Fig. 1) joined *H. segnis* H115, and therefore H116 was transferred to phenon 4. A few strains were haemolytic or showed greening on blood agar.

Phenon 5 (the 'parainfluenzae' cluster) contained four V-requiring strains of *H. parainfluenzae* and two *H. gallinarum* strains. The clustering in this area was not very satisfactory. Since strain H106 moved from the ungrouped strains into phenon 5 in the pattern coefficient dendrogram, it was transferred to phenon 5. It was, however, a duplicate of strain H93 which was in phenon 4 and remained there in the pattern coefficient dendrogram. It may be that phenons 4 and 5 are not distinct, because in the pattern coefficient dendrogram these two phenons were scarcely separated. Strains H91, H96 and H104 were all replicates of the same strain (the last received from a different source), and they were quite closely grouped in both S_G and S_p dendrograms (see Fig. 1).

Phenon 6 (the 'avium' cluster) consisted of four *H. avium* strains (including the type strain), three V-requiring *H. gallinarum* strains and three *H. parasuis* strains. The composition of the phenon was the same in the pattern coefficient dendrogram. No strains were haemolytic but several showed greening.

Phenon 7 (the 'Actinobacillus-Pasteurella' cluster), with phenons 10 and 11, represented strains of *Pasteurella* and *Actinobacillus*. The clustering in this region of the phenogram was poor, and further study of strains of these genera would probably show that these three phenons were more closely allied than is indicated by Fig. 1. Thus, H130 and H131 were

Table 4. *Characteristics of the phenons (percentage of strains positive)*

Test no.	Character	Phenon											
		1	2	3	4	5	6	7	8	9	10	11	12
1	Gram reaction weak or variable	0	0	0	0	0	0	0	0	0	0	0	50
2	Cells in chains and filaments	66	90	55	71	71	60	67	33	33	75	100	0
3	Long rods (>5 µm)	2	20	0	12	14	0	33	0	0	25	0	50
Colonial characters:													
4	Diameter >1 mm	84	70	100	71	100	80	100	0	83	100	100	67
5	Convex	16	10	20	12	14	0	0	25	17	0	0	33
6	Granular	2	20	0	12	43	0	67	0	17	25	0	17
7	Rough	0	0	0	6	0	0	0	0	17	0	0	17
8	Irregular edge	5	0	0	0	0	0	0	25	17	0	0	50
9	Yellow	7	50	5	0	14	100	33	0	0	0	0	17
10	White	20	20	25	0	0	10	67	0	0	50	0	50
11	Opaque	82	90	85	59	100	100	100	0	50	100	50	50
12	Viscous	2	0	0	12	14	0	0	0	0	0	0	0
Growth characters:													
13	At 4 °C	5	0	0	0	0	0	0	0	0	0	0	50
14	At 25 °C	100	100	100	100	100	100	100	100	67	100	100	100
15	At 30 °C	100	100	100	100	100	100	100	100	100	100	100	67
16	At 37 °C	100	100	100	100	100	100	100	100	100	100	100	50
17	At 45 °C	11	0	10	12	14	90	0	0	0	0	50	0
18	Stimulatory effect of CO ₂	2	10	5	6	0	0	0	17	0	0	0	0
19	Anaerobic growth	93	100	80	94	100	100	100	100	100	0	100	100
20	With 0% (w/v) NaCl	98	80	90	100	86	50	67	0	100	50	100	100
21	With 0.5% (w/v) NaCl	100	100	95	100	100	100	100	100	100	75	100	100
22	With 1% (w/v) NaCl	100	100	100	100	100	100	100	0	100	100	100	100
23	With 3% (w/v) NaCl	73	10	55	24	57	100	33	0	0	25	100	83
24	With 5% (w/v) NaCl	0	0	5	0	0	0	33	0	0	0	0	33
25	With 10% (w/v) NaCl	0	0	0	0	0	0	33	0	0	0	0	0
26	X required	93	0	0	6	0	0	0	0	17	0	0	0
27	V required	100	100	95	100	100	100	0	92	17	0	0	0
28	Coccarboxylase required	0	0	0	0	0	0	0	0	0	0	0	33
29	Conversion of δ-ALA to porphyrins	5	100	95	94	86	100	100	92	100	100	100	100
β-Haemolysis of:													
30	Horse blood	0	0	42	6	0	0	0	ND	0	0	0	0
31	Sheep blood	3	0	61	0	14	0	0	0	0	0	0	0
32	Rabbit blood	5	0	63	25	0	0	0	ND	0	0	0	0

Survival at 56 °C:												
33 For 5 min	80	60	55	94	100	100	100	33	67	100	100	83
34 For 10 min	52	30	10	65	100	100	100	8	50	100	0	83
35 For 15 min	23	10	0	18	43	100	33	8	50	100	0	83
36 For 20 min	18	10	0	0	14	60	0	0	50	100	0	83
Growth on:												
37 Horse blood agar	100	100	100	100	100	100	100	100	80	100	100	100
38 MacConkey agar	7	10	5	6	14	0	67	0	40	100	100	67
39 Nutrient agar	11	20	5	6	86	10	100	0	60	100	100	67
Biochemical characters:												
40 Alkaline phosphatase	100	100	100	100	100	100	67	33	100	75	100	0
41 Esterase	86	10	20	29	14	70	0	0	17	25	100	33
42 Esterase lipase	2	10	0	0	0	60	100	0	0	25	50	67
43 Lipase	0	0	0	0	0	0	0	0	0	0	0	0
44 Leucine arylamidase	30	30	20	0	14	50	33	8	0	0	0	0
45 Valine arylamidase	0	0	0	0	0	0	0	0	0	0	0	0
46 Cysteine arylamidase	0	0	0	0	0	0	0	0	0	0	0	0
47 Trypsin	2	0	0	0	0	0	0	0	0	0	0	0
48 Chymotrypsin	0	0	0	0	0	0	0	0	0	0	0	17
49 Acid phosphatase	98	100	100	100	100	100	67	33	100	75	100	17
50 Phosphoamidase	98	100	100	100	100	100	67	17	0	75	100	0
51 α -Galactosidase	0	30	0	0	0	0	0	0	0	0	0	0
52 β -Galactosidase	0	50	0	12	0	0	0	0	17	0	100	0
53 β -Glucuronidase	0	0	0	0	0	0	0	67	0	0	0	0
54 α -Glucosidase	0	0	5	6	0	0	0	0	0	0	0	0
55 β -Glucosidase	0	0	0	0	0	0	0	0	0	0	0	50
56 <i>N</i> -Acetyl- β -glucosamidase	0	0	0	0	0	0	0	0	0	0	0	0
57 α -Mannosidase	0	0	0	0	0	0	0	0	0	0	0	0
58 α -Fucosidase	0	90	0	12	0	0	0	0	0	0	0	0
59 Urease	43	0	45	18	0	0	0	0	0	0	50	0
60 Kovacs' oxidase	75	100	100	12	86	60	100	100	0	75	100	33
61 Catalase	39	100	5	82	100	60	67	0	0	75	50	50
62 Nitrate reduction	100	100	100	100	57	100	100	100	100	75	100	100
63 ONPG test	14	40	95	41	71	10	0	0	50	25	100	0
64 Methyl red	0	0	0	0	0	0	0	0	0	0	0	67
65 Voges-Proskauer	45	20	70	24	0	40	100	0	0	25	100	67
66 Indole	59	20	15	29	0	0	0	58	0	25	0	0
67 H ₂ S from peptone	7	0	47	53	100	70	0	17	40	0	50	17
68 Hugh & Leifson test: acid produced aerobically	49	10	75	53	86	80	33	0	50	0	50	83
69 Hugh & Leifson test: acid produced anaerobically	53	0	70	53	100	90	67	8	33	0	0	83

Table 4. (*cont.*)

Test no.	Character	Phenon											
		1	2	3	4	5	6	7	8	9	10	11	12
Acid production from:													
70	Glycerol	27	20	15	41	29	10	0	8	67	75	100	100
71	Erythritol	2	0	0	0	0	10	0	8	0	0	50	0
72	D(-)-Arabinose	7	10	0	0	0	0	0	0	0	0	0	17
73	L(+)-Arabinose	5	10	10	6	0	30	0	0	0	0	0	0
74	Ribose	89	70	65	18	43	70	67	83	50	50	100	100
75	D(+)-Xylose	39	10	60	18	43	20	33	8	0	50	100	0
76	L(-)-Xylose	7	0	0	0	0	0	0	0	0	0	0	0
77	Adonitol	2	0	0	0	0	0	0	0	0	0	0	0
78	Methyl xyloside	0	0	0	0	0	0	0	0	0	0	0	0
79	Galactose	64	50	65	82	100	60	67	33	100	100	100	0
80	D(+)-Glucose	82	80	90	100	100	100	100	50	100	100	100	100
81	D(-)-Fructose	27	80	90	100	100	100	100	42	100	100	50	100
82	D(+)-Mannose	36	70	95	88	100	90	100	50	100	75	100	100
83	D(-)-Sorbitose	0	10	0	0	0	0	0	0	0	50	0	0
84	Rhamnose	11	0	0	0	0	0	0	8	0	0	0	0
85	meso-Inositol	0	20	5	0	0	40	0	0	0	50	100	100
86	Mannitol	11	40	60	0	71	40	33	25	17	25	100	100
87	Sorbitol	18	10	15	6	29	20	33	25	0	0	0	100
88	Methyl D-mannoside	2	0	0	0	0	0	33	0	0	0	0	100
89	Methyl D-glucoside	2	10	15	0	0	10	0	17	50	0	0	100
90	N-Acetylglucosamine	73	50	55	94	100	100	100	42	83	75	100	100
91	Amygdalin	0	10	10	0	0	10	0	8	0	0	0	100
92	Arbutin	11	20	10	0	0	20	33	0	33	25	0	100
93	Aesculin	9	10	10	0	14	20	33	33	17	25	0	100
94	Salicin	0	10	10	0	14	20	33	0	0	0	0	100
95	D(+)-Cellobiose	11	20	15	0	14	30	33	17	17	0	0	100
96	Maltose	59	70	75	100	100	70	100	58	100	100	100	100
97	Lactose	11	30	35	29	29	30	0	17	100	75	100	50
98	D(+)-Melibiose	9	0	0	6	0	10	0	17	0	0	50	0
99	Sucrose	14	70	90	100	100	100	100	25	100	100	100	100
100	D(-)-Trehalose	14	30	30	18	100	70	67	33	100	100	100	100
101	Inulin	0	20	5	0	0	10	0	8	0	0	0	100
102	D(+)-Melezitose	32	50	55	53	57	80	33	33	100	75	100	100
103	D(+)-Raffinose	0	30	40	29	43	70	0	0	100	25	50	0
104	Dextrin	0	0	55	59	43	0	0	25	100	0	100	100
105	Starch	5	10	50	29	57	10	33	25	20	50	100	100
106	Glycogen	5	0	35	24	14	10	0	0	17	0	50	0
107	Alkaline medium with dulcitol	0	0	0	6	0	0	33	0	0	0	0	0

Sensitivity to:													
108	Tetracycline	100	100	94	100	80	100	100	100	100	100	100	100
109	Chloramphenicol (50 µg)	100	100	100	100	100	100	100	100	100	100	100	100
110	Erythromycin	100	100	100	100	100	100	100	100	100	100	100	100
111	Sulphafurazole (500 µg)	55	80	25	18	14	40	33	92	50	25	50	50
112	Novobiocin	98	100	25	94	100	70	67	100	100	25	0	100
113	Oleandomycin	43	80	10	18	57	20	33	58	33	50	50	50
114	Penicillin G (5 units)	95	100	100	76	43	20	100	100	100	100	100	17
115	Streptomycin (25 µg)	98	100	100	100	100	100	100	67	100	100	100	33
116	Co-trimoxazole	84	100	85	76	86	80	67	92	83	75	100	33
117	Gentamicin	100	100	90	100	100	100	100	100	100	100	100	100
118	Cephaloridine	91	100	95	88	86	70	100	100	100	100	100	100
119	Colistin sulphate	95	100	95	100	86	100	100	83	100	100	100	50
120	Ampicillin	100	100	95	76	71	80	100	100	100	100	100	33
121	Carbenicillin	98	100	100	76	71	80	100	100	100	100	100	50
122	Streptomycin (10 µg)	98	90	100	100	43	90	100	58	100	100	100	17
123	Oxytetracycline	98	100	100	65	100	0	33	92	100	100	50	83
124	Chlortetracycline	100	100	100	71	100	10	100	100	100	75	100	100
125	Chloramphenicol (10 µg)	100	100	100	100	100	100	100	92	100	100	100	100
126	Furazolidone	100	100	100	100	100	100	100	100	100	100	100	100
127	Sulphafurazole (50 µg)	43	50	25	24	14	0	0	58	50	25	100	17
128	Neomycin	98	90	100	100	43	90	100	92	83	100	100	67
129	Penicillin G (1.5 units)	70	90	95	47	71	20	100	100	83	75	100	17
130	Resistance to tellurite	34	0	5	0	0	0	0	0	40	0	0	67
131	Resistance to azide	89	70	95	94	86	100	100	100	40	100	50	67
132	Growth on 0.1 % phenol	68	10	90	94	86	100	67	75	40	0	100	83
133	Mucate	95	70	95	53	29	86	67	58	100	25	0	0
134	Gluconate	73	80	70	41	86	100	100	58	83	100	0	100
135	Lipase	55	70	50	35	43	90	0	25	100	50	0	0
136	Tetrathionate reductase	91	60	85	59	14	80	100	67	50	0	0	0
137	Pectate	0	0	5	0	29	0	0	0	0	0	100	0
138	Citrate	7	0	0	6	14	0	0	0	0	50	100	0
139	Malonate	5	0	0	0	29	0	0	0	0	25	0	0
140	Acetate	0	0	0	0	0	0	0	0	0	0	0	0
141	Stimulation of growth in air by 0.5 % NaCl	27	10	5	27	14	56	33	100	33	0	0	0

ND, Not determined.

All strains were non-motile and DNase negative; none hydrolysed gelatin, tyrosine, xanthine, casein or aesculin, or produced hydrogen cyanide, or produced acid from dulcitol or amylose. These characters were therefore omitted from the computer analysis.

Results of tests for utilization of compounds as carbon/energy source were on media containing accessory growth factors.

derived from the same strain, yet the former was in phenon 11 and the latter in phenon 7. One strain, H133, seemed to be closest on the basis of the pattern coefficient to phenon 10 and was transferred to that group. Strain H148, received as *H. influenzae-murium* (Csukás, 1976), appeared more like a strain of *Pasteurella* than *Haemophilus* and did not require X or V factors. Moreover, it identified as *P. multocida* using the matrix of Bascomb *et al.* (1973).

Phenon 8 (the 'somnus' cluster) contained strains which required V alone. They were divided into two subgroups, corresponding to *H. paragallinarum* (8a) and *H. somnus* (8b), together with one strain of *H. segnis* (H116). If more strains had been included these subclusters may have been well-separated species. The strains in phenon 8 were characterized by a requirement for a small concentration of NaCl for growth in air or in 5–10% CO₂ in air, grew slowly, and gave pinpoint colonies on chocolate agar at 37 °C after 24 h incubation. In the pattern coefficient dendrogram the four strains of *H. paragallinarum*, though remaining a tight cluster, were a distant satellite group to phenon 1, and thus separated from the cluster of *H. somnus* strains (which remained together). Two strains of *H. segnis* became a satellite of phenons 3 and 4. These differences are probably due to the slow growth of the strains of phenon 8. Ungrouped strains that were loosely associated with phenon 8 were *H. vaginalis* H65, a slow-growing strain of *H. paragallinarum* (H114), two strains of *H. haemoglobinophilus* (H18 and H20), and strains of *H. haemolyticus* (H23) and *H. influenzae* (H54).

Phenon 9 (the 'aphrophilus' cluster) contained five strains of *H. aphrophilus* including the type strain H9. None of these required X or V for growth. Also included in both the *S_G* and *S_P* phenograms was a strain (H8) received as *H. influenzae* biotype III which appeared to require both X and V, but was δ -ALA positive. Most strains showed some greening, but none was haemolytic.

Phenon 10 (the 'Pasteurella' cluster) contained the three remaining *Pasteurella* strains. Two ungrouped strains attached to it were *H. haemoglobinophilus* H17 and *H. parainfluenzae* H106 which was moved to phenon 5 (see above).

Phenon 11 (the 'Actinobacillus' cluster) contained the remaining strains of *Actinobacillus*.

Between phenon 11 and phenon 12 there were 15 ungrouped strains received under various names (Fig. 1). Most of these remained in the ungrouped residue near the foot of the dendrogram derived from the pattern coefficient. They are considered further in the Discussion.

Phenon 12 (the 'piscium' cluster) contained all the strains from fish except for the carp strain H158, which was ungrouped. The two cocarboxylase-requiring strains of *H. piscium* (H159, H160) clustered separately from the four Weymouth fish isolates which did not require cocarboxylase. It is possible that if more strains had been available there would have been two distinct groups. Three strains appeared to be weakly Gram-positive or Gram-variable, a characteristic noted by Kilian (1976*a*).

General features of the pattern coefficient dendrogram

The differences between the *S_G* and *S_P* dendrograms as they relate to single strains have been mentioned above. The general features of the *S_P* dendrogram (Fig. 3) merit brief mention. The phenons in Fig. 1 were substantially the same in the *S_P* dendrogram, as would be expected from the rather uniform values for vigour in Table 3. Phenons 4 and 5 were partly intermingled and phenon 8 appeared as two separate groups, *H. paragallinarum* and *H. somnus*, as noted above. The relationships of the major groups in the two dendrograms were somewhat different: these probably have little significance (see Discussion).

Characteristics of the phenons

The characteristics of the 12 phenons are shown in Table 4. It was not possible to find a set of tests that permitted unambiguous separation of all the phenons. The most discriminating tests in Table 4 are: test 9, yellow pigment; 17, growth at 45 °C; 22, growth on 1% NaCl; 26,

requirement for X; 27, requirement for V; 28, requirement for cocarboxylase; 53, β -glucuronidase; 58, α -fucosidase; 60, oxidase; 61, catalase; 63, ONPG; 86, acid from mannitol; 97, acid from lactose; 99, acid from sucrose; 100, acid from trehalose.

DISCUSSION

The groupings are not as clear-cut as those often found in numerical taxonomic studies. A partial explanation may be the level of test reproducibility, though the value of 5% found in this study is not particularly high (Sneath, 1974; Jones, 1975; Wilkinson & Jones, 1977; Goodfellow *et al.*, 1979; Austin *et al.*, 1979). A more likely reason is the fastidious growth requirements of the bacteria. Not all strains gave clear-cut acidity from glucose in Hugh & Leifson or API 50E tests, possibly due to suboptimal growth. The influence of this on numerical taxonomy has not been explored, but one would expect that strains that were unable to manifest their full metabolic potential would commonly fall outside the tight clusters formed by strains that were growing under better conditions. Alternatively, the strains which appeared as satellite or ungrouped strains could have been misidentified so that the names under which they were received were erroneous. It is difficult to know which explanation is most likely; some comments on individual strains are noted below. The explanation is not simply due to differences in vigour, because results with the pattern coefficient affected only a minority of the poorly-clustered strains.

Although the nucleus of the phenon 1 cluster was composed of classical *H. influenzae*, its internal structure was not very clear. Kilian (1976*a*) divided *H. influenzae* into five biotypes on biochemical and morphological properties. In the present study these biotypes were not well separated but it should be noted that comparatively few strains from the different biotypes were included. There is some evidence that the biotypes are not entirely arbitrary groupings, e.g. Kilian *et al.* (1979) found that 93% of strains causing meningitis belonged to biotype I, whereas this biotype is uncommon in the respiratory tract. Our findings do not provide much evidence on the relationships of the capsular serotypes, but Kilian *et al.* (1979) noted that there may be a clinically significant correlation between biotype and serotype. Strain H128 was a satellite to the main core of *H. influenzae*, in a similar position to that found in the study of Sneath & Johnson (1973).

Haemophilus aegyptius has been differentiated from *H. influenzae* on the basis of its haemagglutinating ability, but this feature is not particularly characteristic of strains causing acute conjunctivitis (Smith, 1954; Kilian *et al.*, 1976). We examined a few strains, using human blood by the method of Davis *et al.* (1950). Our findings were similar to Kilian's in that some strains of several phenons, but not all conjunctival strains, were positive. The strains in the small subcluster (also observed by Sneath & Johnson, 1973) which were received under the name *H. aegyptius* tended to ferment fewer carbohydrates than typical strains of *H. influenzae*. Kilian (1976*a*) found that *H. aegyptius* strains belonged mainly to his biotype III, but several strains of that biotype are found elsewhere in Fig. 1.

Haemophilus haemolyticus was originally separated from *H. influenzae* because it was haemolytic. Kilian (1976*a*) considered it to be a separate species, but the present study suggests they are haemolytic variants. In the study of Sneath & Johnson (1973) strain H21 was found to require X and V for growth, but in Kilian's work (1976*a*) and the present study, it required V only for growth and gave a pink fluorescence in the δ -ALA test typical of X-independence. However, the peculiarities of haemolysis noted in this study and in that of Sneath & Johnson (1973) point to the fact that it is not just a contaminated culture; the strain gave weak β -haemolysis close to the colony and greenish discoloration of the blood around the β -haemolytic zone. White & Granick (1963) showed certain strains had enzymic activities typical of X-independent strains but were unable to grow on media totally devoid of haematin. Kilian (1976*a*) suggested this was the explanation for the growth requirements determined by Sneath & Johnson (1973).

Four of the six strains of *H. haemoglobinophilus* (H16, H17, H18, H20) required only X for growth as expected, but they, with H19, were all among the ungrouped strains and did not cluster together. Strain H19 required no growth factors; it may be an X-independent mutant. The type strain H15 in this study appeared to require both X and V, in contrast to the findings of Sneath & Johnson (1973), but was in phenon 1 in both studies. It may be that several species of *Haemophilus* occur in dogs, and these organisms certainly need further study.

The three strains of *H. paraphrohaemolyticus* all required only V, but two of them (including the type strain H87) have lost their haemolytic ability and appeared as peripheral members of phenon 1. The third (H89) occurred in phenon 4, which would seem to be a more likely position for strains of this species. Nevertheless, the strains in phenon 1 (Fig. 1) were still members of that cluster in the S_p dendrogram. This species likewise deserves further investigation. These strains had adapted to growth in air without CO₂.

Kilian (1976*a*) proposed a new species *H. segnis* with a GC value of 43–44 mol %, which is rather high for the genus. The status of this species is not clear. The type strain H117 was in phenon 1 (but was ungrouped in the S_p dendrogram) whereas the other strains were in phenon 4 or phenon 8 (Fig. 1). All required only V factor, and gave results in reasonable accord with those of Kilian & Theilade (1978).

Phenon 2 consisted of one of the groups of porcine strains (others were in phenon 6), but though the species *H. suis* was described as requiring X and V (Lewis & Shope, 1931), the surviving cotype H59 (if it is authentic) requires only V (Sneath & Johnson, 1973; Kilian, 1976*a*). It is now the type strain of *H. parasuis* in the *Approved Lists* (Skerman *et al.*, 1980). Swine strains requiring both X and V have, however, been isolated recently (Biberstein *et al.*, 1977). The organism associated with enteritis of rabbits (Targowski & Targowski, 1979) resembles strains of phenon 2 in many ways.

Phenons 3, 4 and 5 were composed of V-requiring strains from the respiratory tracts of various animals. Perhaps the three clusters should be combined, but there are indications that they may be best considered as separate groups. Phenon 3 represented strains that are usually haemolytic and urease positive, which are considered by Biberstein *et al.* (1977) to represent *H. parahaemolyticus*. However, the identity of this species, in view of the discrepant position of what are thought to be different stocks of the type strain (H21, H149) is dubious. Some are from swine (including those labelled *H. pleuropneumoniae*, which do not seem to be distinct from the others): unfortunately the type strain of *H. pleuropneumoniae* was not studied.

Phenons 4 and 5 were partly intermingled in the pattern coefficient phenogram, which shows their close relationship. Most strains of phenon 4 correspond to *H. parainfluenzae*; *H. paraphrophilus* does not seem to be distinct from them, so this may represent variants that require CO₂. Strain H24 is a haemolytic strain with unusual growth requirements. It needed both X and V for growth but was able to convert δ -aminolaevulinic acid to porphyrin. It may perhaps lack the enzyme needed to chelate iron into the porphyrin.

Apart from three swine strains, phenon 6 represented *H. avium* strains; these differ from *H. paragallinarum* in not producing coryza in chickens (Hinz & Kunjara, 1977). Whether strains H109 and H111, received as *H. gallinarum*, produce coryza is not known, but they seem likely to be strains of *H. avium*. This phenon is taxon C of Kilian (1976*a*).

It is not clear why phenons 7, 10 and 11 (*Actinobacillus* and *Pasteurella*) did not form one cluster as in the study of Sneath & Johnson (1973), but the present findings do emphasize the relatedness of these two genera to *Haemophilus*.

As noted earlier, phenon 8 may well represent two species, *H. somnus* and *H. paragallinarum*. The results for *H. somnus* are in good agreement with those of Bailie *et al.* (1973); this species, though not validly published, is associated with a characteristic disease of cattle (thromboencephalomyelitis) and its phenotypic peculiarities suggest it is a good taxon. The four strains of *H. paragallinarum* (including the type strain H144) produce coryza in

fowls (Hinz & Kunjara, 1977) and again this, with the phenotypic properties of phenon 8, suggests it is also a good taxon.

The satellite strain H54 was a member of biotype V of *H. influenzae* in the classification of Kilian (1976*a*). It required both X and V and was δ -ALA negative, and we are unable to explain its position as an ungrouped strain in both the S_G and S_p phenograms.

The growth requirements of the strains of *H. aphrophilus* in phenon 9 require comment. There are conflicting reports on the X-requirement of this species (Khairat, 1940; Sutter & Finegold, 1970; Sneath & Johnson, 1973). Kilian (1976*a*) found it was able to convert δ -aminolaevulinic acid to porphyrin, as we found in the present study. It is possible that *H. aphrophilus* readily mutates to X-independence (Boyce *et al.*, 1969), or these strains may lack the power to readily chelate iron into the porphyrin. *Haemophilus aphrophilus* also adapts readily to growth in air without added CO₂ (Khairat, 1940). We did not find strain H10 was particularly different from H9 as implied by the report of the Subcommittee on the Taxonomy of *Haemophilus* (Biberstein & Zinnemann, 1971).

All the strains from fish were found in phenon 12 except for that from carp erythrodermatitis (H158). If phenon 12 is considered to be one species, *H. piscium*, then a requirement for cocarboxylase is not a constant feature. The low similarity (65%) with other species of *Haemophilus* suggests that it may be best excluded from the genus, but where it should go is not clear.

The ungrouped strains between phenons 11 and 12 were received under a variety of names. Only H16, H122 and H129 required X, V or both. Several showed haemolysis or greening. The pattern coefficient computation suggested that H122 might be an aberrant strain of phenon 2, which would be in accord with its V-requirement and porcine origin. Strain H16 was X-requiring and δ -ALA negative, as found by Sneath & Johnson (1973), but did not group (in either phenogram) with H15 as in that study. Strain H129 appears to be an aberrant member of *Haemophilus* and may possibly represent a new species of this genus. We found it was δ -ALA negative, but required V as well as X. Kilian (1976*a*) found it needed X only, and Ryan (1968) believed it did not require V, though he noted slight satellitism around V-producing staphylococci. We found, unlike Ryan (1968), that nitrate was reduced; other properties were in good agreement.

The present groupings agree quite closely with those obtained by Sneath & Johnson (1973) where strains were common to the two studies. Thus, the core of strains of *H. influenzae* in phenon 1 and the peripheral position in that phenon of H1, H15, H21, H34 and H128 are evident in both surveys. Similarly, strains H109, H110, H111 and H125 form a tight cluster in both. The studies differ in the greater diversity in the V-requiring strains; the small number of strains in the earlier study may account for much of this variation. The principal difference in broad groupings is the scatter amongst strains of *Actinobacillus* and *Pasteurella* in the present investigation.

This study also agrees reasonably well with that of Kilian (1976*a*). Many of the phenons correspond to groups recognized by Kilian: phenon 1 is *H. influenzae*, 2 is *H. parasuis*, 3 is mainly *H. parahaemolyticus*, 5 is probably *H. parainfluenzae* biotype I, 6 is taxon C, 8a is possibly *H. paragallinarum*, 9 is *H. aphrophilus* and 12 is *H. piscium*. However, we did not find clear groupings that correspond to *H. haemolyticus*, *H. segnis* or *H. paraphrophilus*. The disagreements on individual tests were rather few when the different test methods are taken into account. Our tests for catalase, oxidase, and carbohydrate fermentations were evidently rather insensitive; these tests account for many of the discrepancies. We did, however, obtain the opposite result from Kilian in some instances that seemed unlikely to be due to these factors or the sets of strains: thus phenon 2 was oxidase positive; phenon 6 was α -glucosidase negative; phenon 12 produced acid from mannitol and reduced nitrate.

The relation of the phenons to serology is difficult to assess, as the data are fragmentary. The relative serological homogeneity of *H. influenzae* has long been known (Pittman, 1931), and Nicolet (1971) has shown similar homogeneity in *H. parahaemolyticus*; in both cases

serological subgroups have been recognized which are concordant with the clusters found here. The grouping of most strains of *H. paraphrophilus* in phenon 4 is congruent with its serological homogeneity (Frazer *et al.*, 1975) and the heterogeneity of *H. paraphrohaemolyticus* is reflected in the scattered occurrence of the strains in Fig. 1. Too few strains of *H. aphrophilus* and *H. haemoglobinophilus* were studied for comparison with the serological data of Frazer *et al.* (1975). The grouping of *H. somnus* strains agrees with the serological homogeneity reported by Shigidi & Hoerlein (1970).

The present findings emphasize that host animal is an inadequate basis on which to establish species of *Haemophilus*. One animal can carry several different species, and it seems likely that strains of one phenon can sometimes be found in several animals. Growth factor requirement is also an insecure basis for speciation, particularly in the strains that require only V. Similarly, little emphasis should be placed on haemolysis or CO₂ requirement. However, a few phenons are linked to a characteristic disease (see Nicolet, 1968, 1971; Kilian, 1976*a, b*, for discussion).

A matter of considerable practical importance is the extent to which the groups within the genus *Haemophilus* can be distinguished. The pattern of variation in our results is not so clear-cut as is seen in some bacterial genera, and it has not been possible to find a small set of highly discriminatory tests. There are two ways in which this aspect can be explored – by estimating the overlap between the phenons statistically (Sneath, 1980), and by using the phenon descriptions as a data base against which to identify new isolates by numerical methods such as those of Bascomb *et al.* (1973). It is hoped to carry out further work on these lines in the near future.

Although little is known in detail about the patterns of taxonomic variation in different groups of bacteria, there is some evidence that the variation in parasitic groups is much more complex than in predominantly free-living forms (e.g. *Streptococcus*, phytopathogenic pseudomonads: Jones, 1978; Palleroni, 1975). It should also be remembered that some of the apparent misplacements of individual strains may be due to the occurrence of hybrids, of unusual plasmids and the like. Plasmids are reported as common in *H. influenzae* (Stuy, 1979), though the extent to which they affect the phenetic position of bacteria is still poorly known. Alternatively, some apparently aberrant strains (e.g. H54, H129) may be the nuclei of hitherto unrecognized new species.

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