Taxonomy of *Bordetella* and Related Organisms of the Families *Achromobacteraceae*, *Brucellaceae*, and *Neisseriaceae*

R. JOHNSON and P. H. A. ŠNEATH

*Computer Trials Laboratory, Central Public Health Laboratory, Colindale, London N.W.9, England,
and Medical Research Council Microbial Systematics Unit, University of Leicester,
Leicester, England*

A numerical taxonomic study was made of strains belonging to the genera *Bordetella*, *Alcaligenes*, *Brucella*, *Acinetobacter*, *Moraxella*, *Neisseria*, *Haemophilus*, *Actinobacillus*, and *Pasteurella*, together with a few strains from allied genera. They were examined for 139 characters covering a wide range of tests and properties. The strains fell into two major groups. Group A contained *Haemophilus*, *Actinobacillus*, and *Pasteurella*. Group B contained eight main clusters, representing, respectively, *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica* combined with *Alcaligenes faecalis*, *Brucella*, *Acinetobacter anitratus*, *Acinetobacter lwoffii*, and two clusters containing *Moraxella* and *Neisseria* strains. *Brucella* and the three clusters of *Bordetella* (including *Alcaligenes*) were more similar to one another than to the other clusters; it is suggested that the family *Brucellaceae* should comprise these three genera, and that the other genera should be placed elsewhere.

Several families of bacteria listed in *Bergey's Manual* (13) represent aggregations of genera which at best can only be described as loose associations. Instead of encompassing organisms which overall are closely similar, they have been set up to accommodate genera with one or two prominent characters in common. Such a group is *Brucellaceae*, or its predecessor *Parvobacteriaceae* (12, 63). This family is the niche for those gram-negative, rod-shaped bacteria which require rich media for growth and are responsible for many human and animal infections. Their potential pathogenicity and the alleged difficulties of working with them account largely for their neglect by taxonomists. They tend to grow poorly or to die out on the usual media, whereas the addition of blood to increase growth often interferes with tests.

Within *Brucellaceae* the genus *Bordetella* Moreno-López 1952 has been one of the least studied. Three species are generally recognized: the type species *B. pertussis* (the whooping-cough bacillus), *B. parapertussis*, and *B. bronchiseptica*. Although these organisms have been placed in various genera (e.g., *Haemophilus*), in recent years their segregation as a separate genus has become generally accepted (13, 84). They do not require X factor (haematin) or V

factor (nicotinamide adenine dinucleotide) as do species of *Haemophilus*. The only reason that *B. pertussis* requires blood is to remove toxic substances from growth media; the blood can be replaced by starch or charcoal (65) or by anionic-exchange resins (45).

Bordetella species are obligate aerobes, whereas species of *Haemophilus* are facultative anaerobes. The guanine plus cytosine (G + C) ratios also differ, being 67 to 70% for *Bordetella* and 38 to 42% for *Haemophilus* (20, 36, and findings reported below).

The separation of *Bordetella* from *Alcaligenes*, *Acinetobacter*, and *Brucella* is more difficult to justify, and the main motive for the present work was to investigate this question. Many workers have noted points of similarity between *Bordetella bronchiseptica* and *Alcaligenes faecalis* in particular, although they differ somewhat in G/C ratio (20). There have been suggestions (9, 10, 83) that *Bordetella bronchiseptica* should be placed in either the genus *Brucella* or *Alcaligenes* whereas Steel and Cowan (76) considered that *Bordetella parapertussis* should be included in *Acinetobacter*. However, there has not been any convincing evidence on these relationships from either serological or genetic studies. We have therefore

compared *Bordetella* with some related organisms chosen from the family *Brucellaceae*, or from genera in which *Bordetella* species have been placed from time to time. The main findings, together with details on *Bordetella* and its close allies, are presented here; detailed findings on the genera *Haemophilus*, *Pasteurella*, and *Actinobacillus* are presented elsewhere (72). The individual strain results are also on record (R. Johnson, Ph.D. thesis, Univ. of Leicester, England, 1972).

MATERIALS AND METHODS

Bacterial strains. One hundred fifty-six strains were selected for study (Table 1). Thirty-eight additional strains of the genera *Haemophilus*, *Pasteurella*, and *Actinobacillus* were also studied and are reported on elsewhere (72). Type strains listed by Sneath and Skerman (73) and the American Type Culture Collection (2, 3) were included as far as possible; about forty recent isolates were also examined.

On receipt, all strains were checked for purity and freeze-dried after the fewest possible number of subcultures. Stock cultures were subcultured twice weekly. A careful watch was kept for rough variation or other change, and if this was seen, or contamination occurred, the stock cultures were discarded and replaced from freeze-dried cultures. Stock cultures were replaced in any event after about 25 transfers.

Media. Three maintenance media were used. Most strains were maintained on serum agar. This contained nutrient broth no. 2 (Oxoid Co.) (25 g), agar no. 1 (Oxoid) (11 g), nicotinic acid (20 mg), distilled water (930 ml). The pH was adjusted to 7.2, and, after autoclaving, the medium was held at 50 C and the following sterile solutions were added: haematin (0.05% wt/vol) (5 ml), nicotinamide adenine dinucleotide (NAD) (0.05% wt/vol) (5 ml), cysteine hydrochloride (2.5% wt/vol) (10 ml), inactivated horse serum no. 5 (Burroughs Wellcome Co.) (50 ml). After careful mixing, the medium was dispensed in petri dishes. It was necessary to add these last four components to many of the media used in this study (to avoid repetition they will be referred to as mixture A). The haematin solution was prepared by the method of Butler (15) and sterilized by autoclaving for 5 min at 121 C. The solutions of NAD and cysteine hydrochloride were sterilized by filtration. Some strains did not grow well on this medium. *Haemophilus* strains were therefore maintained on heated blood agar (19), and *Bordetella pertussis* strains were maintained on charcoal blood agar (Oxoid).

Serum agar containing haematin and NAD allowed the growth of all strains except those of *Bordetella pertussis*, but to insure comparability of tests a basal medium was developed that gave satisfactory growth of all strains. Many tests require media which must be transparent so that color changes or zones of clearing or opacity can be observed. Following the suggestions of Kuwajima, Matsui, and Kishigami (45) and of Sutherland and Wilkinson (78), it was found that addition of an anionic exchange resin gave a

satisfactory medium that was both transparent and supported the growth of all strains. The basal medium, designated BM agar, had the following formula: nutrient broth no. 2 (Oxoid) (20 g), casein hydrolysate (Oxoid) (5 g), agar no. 1 (Oxoid) (11 g), nicotinic acid (20 mg), KH_2PO_4 (0.5 g), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.4 g), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (10 mg), CaCl_2 (100 mg), Dowex I-X8 (BDH Co.) (5 g), and distilled water (930 ml). The medium was adjusted to pH 7.2 and after autoclaving for 15 min at 121 C was cooled to 50 C before adding mixture A. The complete medium was further cooled to 44 C and carefully mixed before pouring (the agar should set quickly so that the resin remains suspended). BM broth was prepared from the same formula by omitting the agar. The resin must be activated before incorporation into the medium, using the following procedure: 500 g of Dowex I-X8 (20-50 U.S. mesh) is washed in tap water and then covered with 2 liters of 0.5 M NaOH for 2 h with occasional agitation. The resin is then washed repeatedly with distilled water until the pH of the effluent is below 7.5. The final washing is carried out by packing a glass column with the resin and passing distilled water from a 50-liter tank for about 24 h. Results of tests performed using BM as a basal medium showed very good consistency with the results obtained using conventional media.

Stock cultures and tests for all except eleven strains were incubated at 35 C in a moist atmosphere. The following strains grew poorly at 35 C and were incubated at 25 C: J10, J12, J13, J17, J24, J25, J26, J28, J29, and J34. Test results, unless otherwise stated, were recorded after incubation for 2, 5, and 7 days after inoculation from 48-h-old agar cultures, and unless otherwise stated a definite reaction within 7 days was scored as positive in the computer analysis.

Because good growth of all strains was not obtained on conventional media, many of the usual test methods had to be modified. As a safety precaution, some pathogenic strains had to be tested separately and with a limited range of tests. Consequently, only 84 characters were recorded for the *Brucella* and *Francisella* strains instead of the 139 characters recorded for the other strains.

Staining and morphology. Strains were grown on BM agar and examined after 1, 3, and 5 days or 2, 3, and 5 days for the slower-growing cultures. Cell shape and arrangement were studied by the hanging drop technique; heat-fixed films were stained by Kopeloff and Beerman's modification of Gram's method (19) and counterstained with 0.1% basic fuchsin in 0.5% aqueous phenol. Colonial morphology was described from 48-h-old cultures on BM agar. Motility was determined from hanging-drop preparations examined daily up to 14 days after incubating cultures in duplicate at 25 and 35 C in Craigie tubes (18) of BM broth containing 0.25% Davis New Zealand agar.

Cultural properties. Ability to grow anaerobically was determined by inoculating a loopful of 48-h-old broth culture onto BM agar and incubating it in an anaerobic jar containing hydrogen and a catalyst. Growth anaerobically in the presence of nitrate was determined similarly on BM agar containing 0.1% (wt/vol) KNO_3 . Ability to grow on nutrient agar was determined on blood agar base (Oxoid). Hemolysis of

TABLE 1. *Strains studied*

Strain no. in present survey	Received as	Source ^a and collection no.
J1	<i>Alcaligenes denitrificans</i>	NCTC 8582 ^b
J2	<i>A. odorans</i>	NCTC 10416 ^b
J3	<i>A. faecalis</i>	NCTC 8764
J4	<i>A. faecalis</i>	NCTC 655
J5	<i>A. faecalis</i>	NCTC 8769
J6	<i>A. faecalis</i>	NCIB 8156
J7	<i>A. faecalis</i>	CCEB 591
J8	<i>A. viscosus</i>	NCTC 3233
J9	<i>A. viscosus</i>	NCIB 8154 ^c
J10	<i>A. viscosus</i>	NCIB 8596
J11	<i>A. metalcaligenes</i>	NCIB 9018
J12	<i>A. metalcaligenes</i>	NCIB 9021
J13	<i>A. metalcaligenes</i>	NCIB 8734
J17	<i>Agrobacterium tumefaciens</i>	NCPFB 397
J18	<i>Agrobacterium</i> sp.	MJT ZH1
J23	<i>Acinetobacter anitratus</i>	NCTC 8102
J24	<i>A. anitratus</i>	ATCC 14290
J25	<i>A. calcoaceticus</i>	ATCC 23055 ^d
J26	<i>Achromobacter haemolysans</i>	ATCC 17988
J27	<i>Acinetobacter lwoffii</i>	NCTC 5866 ^e
J28	<i>Acinetobacter</i> sp.	MJT F4/6/20
J29	<i>Acinetobacter</i> sp.	Baumann 19
J30	<i>Acinetobacter</i> sp.	M.S.U.
J31	<i>Achromobacter haemolyticus</i> subsp. <i>alcaligenes</i>	NCTC 10306 ^b
J32	<i>A. haemolyticus</i>	NCTC 10305 ^b
J33	<i>A. citroalkaligenes</i>	NCTC 10307 ^b
J34	<i>A. conjunctivae</i>	NCTC 10304 ^b
J35	<i>A. mucosus</i>	NCTC 10303 ^e
J36	<i>A. liquefaciens</i>	ATCC 15716
J37	<i>A. lacticus</i>	NCIB 8208
J38	<i>Acinetobacter anitratus</i>	NCIB 9212 ^f
J39	<i>A. anitratus</i>	NCIB 9019
J40	<i>A. anitratus</i>	NCIB 9301
J41	<i>Achromobacter</i> sp.	NCIB 8250
J42	<i>Bordetella pertussis</i>	NCTC 9797 ^c
J43	<i>B. pertussis</i>	NCTC 8189
J44	<i>B. pertussis</i>	NCTC 8631
J45	<i>B. pertussis</i>	IID 18-323 phase I.
J46	<i>B. pertussis</i>	IID Tohama phase I.
J47	<i>B. pertussis</i>	IID Yamapaki phase I.
J48	<i>B. pertussis</i>	IID Opaki phase I.
J49	<i>B. pertussis</i>	IID Maeno phase I.
J50	<i>B. pertussis</i>	IID Kawarada phase I.
J51	<i>B. pertussis</i>	IID Tohama phase III.
J52	<i>B. pertussis</i>	IID Sakairi phase III.
J53	<i>B. pertussis</i>	IID Sugata phase III.
J54	<i>B. parapertussis</i>	NCTC 5952 ^d
J55	<i>B. parapertussis</i>	ATCC 15237 ^d
J56	<i>B. parapertussis</i>	ATCC 15989 ^d
J57	<i>B. parapertussis</i>	ATCC 9305
J58	<i>B. bronchiseptica</i>	MSU
J59	<i>B. bronchiseptica</i>	NCTC 452 ^d
J60	<i>B. bronchiseptica</i>	NCTC 8344
J61	<i>B. bronchiseptica</i>	ATCC 19
J62	<i>B. bronchiseptica</i>	ATCC 780
J63	<i>B. bronchiseptica</i>	ATCC 785
J64	<i>B. bronchiseptica</i>	ATCC 786
J65	<i>B. bronchiseptica</i>	ATCC 4617

TABLE 1.—Continued

Strain no. in present survey	Received as	Source ^a and collection no.
J66	<i>B. bronchiseptica</i>	ATCC 10580
J67	<i>B. bronchiseptica</i>	ATCC 12714
J68	<i>B. bronchiseptica</i>	ATCC 14064
J69	<i>B. bronchiseptica</i>	ATCC 14065
J70	<i>B. bronchiseptica</i>	ATCC 15101
J71	<i>B. bronchiseptica</i>	ATCC 14455
J72	<i>B. bronchiseptica</i>	IID M12
J128	<i>Moraxella bovis</i>	NCTC 8561 ^c
J129	<i>M. bovis</i>	NCTC 9425
J130	<i>M. bovis</i>	NCTC 9426
J131	<i>M. liquefaciens</i>	NCTC 7911 ^c
J132	<i>M. lwoffii</i>	NCTC 5867
J133	<i>M. lwoffii</i>	NCTC 7976
J134	<i>M. lacunata</i>	NCTC 7784 ^{c,g}
J136	<i>Mima polymorpha</i> var. <i>oxidans</i>	ATCC 10973
J137	<i>Moraxella lacunata</i>	H 7911/SS
J139	<i>M. bovis</i>	H 9425/SS
J140	<i>M. osloensis</i>	H A1920 SS(S) ^b
J141	<i>M. phenylpyruvica</i>	H 2863 SS ^e
J142	<i>M. polymorpha</i>	H(ATCC 17958) ^b
J143	<i>M. kingii</i>	NCTC 10529 (4177/66) ^b
J144	<i>M. ovis</i>	H 199/55 SS
J145	<i>Moraxella</i> sp.	MSU
J146	<i>Neisseria caviae</i>	NCTC 10293 ^b
J147	<i>N. flavescens</i>	NCTC 8263 ^b
J148	<i>N. catarrhalis</i>	NCTC 3622
J149	<i>N. catarrhalis</i>	NCTC 4103
J150	<i>N. pharyngis</i> "flavus"	NCTC 4590
J152	<i>N. pharyngis</i> "siccus"	NCTC 4591
J153	<i>Noguchia granulosis</i>	ATCC 11479
J154	<i>Brucella abortus</i>	Wey 19 biotype 1
J158	<i>B. abortus</i>	Wey 64/51 biotype 9
J159	<i>B. melitensis</i>	Wey Rev. 1 biotype 1
J161	<i>B. suis</i>	Wey Thomson biotype 1
J162	<i>B. suis</i>	Wey 63/258 biotype 1
J165	<i>B. canis</i>	Wey RM6/66
J172	<i>Pasteurella haemolytica</i> T	NCTC 10371
J179	<i>Francisella tularensis</i>	Owen Jap.
J180	<i>F. novicida</i>	Owen U112 ^b
J181	<i>Bordetella pertussis</i>	PHL Leics.
J186	<i>B. pertussis</i>	Lacey
J187	<i>B. pertussis</i>	Lacey H36
J188	<i>B. pertussis</i>	Lacey H52
J189	<i>B. pertussis</i>	Lacey H103
J190	<i>B. pertussis</i>	Lacey H104
J191	<i>B. pertussis</i>	Lacey H105
J192	<i>B. bronchiseptica</i>	Lacey BR2
J193	<i>B. bronchiseptica</i>	Lacey BR4
J194	<i>B. bronchiseptica</i>	Lacey BR58
J195	<i>B. bronchiseptica</i>	Lacey BR62
J196	<i>B. bronchiseptica</i>	Lacey BR88
J197	<i>B. bronchiseptica</i>	Lacey BR106
J198	<i>B. bronchiseptica</i>	Wey P21
J199	<i>B. bronchiseptica</i>	Wey S23
J200	<i>B. bronchiseptica</i>	Wey DB80
J201	<i>B. bronchiseptica</i>	Wey DB82
J202	<i>B. bronchiseptica</i>	Wey DB84A
J203	<i>B. bronchiseptica</i>	Wey DB85
J204	<i>B. bronchiseptica</i>	Wey DB86

TABLE 1.—Continued

Strain no. in present survey	Received as	Source ^a and collection no.
J205	<i>B. bronchiseptica</i>	Wey DB87D
J206	<i>B. bronchiseptica</i>	Wey DB91
J207	<i>B. bronchiseptica</i>	Wey DB92
J208	<i>B. bronchiseptica</i>	Wey DB105 B2
J209	<i>B. bronchiseptica</i>	Wey DB8 V16
J210	Yellow pigmented rod	IID
J211	<i>B. parapertussis</i>	NCTC 10523
J212	<i>B. parapertussis</i>	NCTC 10524
J214	<i>B. parapertussis</i>	Lautrop 4380
J215	<i>B. parapertussis</i>	Lautrop 4840
J216	<i>B. parapertussis</i>	Lautrop 4847
J217	<i>B. parapertussis</i>	Lautrop 4888
J218	<i>B. parapertussis</i>	Lautrop 5725
J219	<i>B. parapertussis</i>	Lautrop 5743
J220	<i>B. parapertussis</i>	Lautrop 5954
J221	<i>B. parapertussis</i>	Lautrop 6002
J222	<i>B. parapertussis</i>	Lautrop 6006
J223	<i>B. parapertussis</i>	Lautrop 6084
J224	<i>B. parapertussis</i>	Lautrop 6137
J225	<i>B. parapertussis</i>	Lautrop 6225
J226	<i>B. parapertussis</i>	Lautrop 6231
J227	<i>B. parapertussis</i>	Lautrop 7806
J228	<i>B. parapertussis</i>	Lautrop 7915
J229	<i>B. pertussis</i>	Lautrop 3234
J230	<i>B. pertussis</i>	Lautrop 3252
J231	<i>B. pertussis</i>	Lautrop 3286
J232	<i>B. pertussis</i>	Lautrop 3210
J233	<i>B. pertussis</i>	Lautrop 3239
J234	<i>B. pertussis</i>	Lautrop 3254
J235	<i>B. pertussis</i>	Lautrop 3255
J236	<i>B. pertussis</i>	Lautrop 3272
J237	<i>B. pertussis</i>	Lautrop 3277
J238	<i>B. pertussis</i>	Lautrop 3281
J239	<i>B. pertussis</i>	Lautrop P.5759
J240	<i>B. pertussis</i>	Lautrop P.5767
J241	<i>B. bronchiseptica</i>	Lautrop AB65
J242	<i>B. bronchiseptica</i>	Lautrop AB108
J243	<i>B. bronchiseptica</i>	Lautrop AB1043
J244	<i>B. bronchiseptica</i>	Lautrop Ole

^a Abbreviations: ATCC, American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md.; Baumann, P. Baumann, Retina Foundation, Boston, Mass.; CCEB, Czechoslovak Collection of Entomogenous Bacteria, Na cvičišti 2, Prague 6; H, S.D. Henriksen, Kaptein W. Wilhelmsen og Frues Bakteriologiske Institut, University of Oslo, Norway; IID, Institute of Infectious Diseases, University of Tokyo, Japan; Lacey, B. W. Lacey, Department of Bacteriology, Westminster Medical School, London; Lautrop, H. Lautrop, Statens Seruminstitut, Copenhagen; MJT, M. J. Thornley, Low Temperature Research Station, Cambridge; MSU, M. R. C. Microbial Systematics Unit, University of Leicester, England; NCIB, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen; NCPPB, National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Fisheries and Food, Plant Pathology Laboratory, Milton Road, Harpenden, Herts, England; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London N.W.9., England; Owen, C. R. Owen, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Mont.; PHL, Leics, Public Health Laboratory, Leicester, England; Wey, Central Veterinary Laboratory, Weybridge, England.

^b Holotype, type status (2, 3, 73).

^c Proposed working type, type status (2, 3, 73).

^d Co-type, type status (2, 3, 73).

^e Neotype, type status (2, 3, 73).

^f Paratype, type status (2, 3, 73).

^g Recent studies (3, 62) suggest it is not suitable as a type strain, but is probably *Moraxella nonliquefaciens*.

horse and sheep blood was tested on BM agar layered over with BM agar containing 10% (wt/vol) horse or sheep blood. Requirement for increased CO₂ concentration was determined by incubating in air with 5% CO₂.

Resistance tests. Tests were carried out on BM agar. Growth on bile salts was tested on media containing 5, 10, or 40% (wt/vol) bile salts (Oxoid); tolerance to sodium chloride was determined on agar containing 0, 0.5, 3.0, 4.5, 6.0, 7.5, or 9.0% (wt/vol) sodium chloride. BM without sodium chloride was prepared from single constituents omitting NaCl; it contained only a small amount of sodium chloride (about 0.04%) derived from the added serum.

Sensitivity to the pteridine compound 0/129 (2, 4 diamino, 6-7 di-iso propyl pteridine) was determined by placing paper disks determined on agar containing 0/129 onto streaked agar plates (the disks were soaked in a 0.4% ethanolic solution of 0/129 and dried at 37 C). Dye sensitivity was tested by the paper strip method (19): paper strips were soaked in aqueous solutions of the following dyes: basic fuchsin (BDH) (5 µg/ml), methyl violet (E. Gurr Ltd.) (2.5 µg/ml), pyronin G (BDH) (1.25 µg/ml), safranin O (BDH) (1.25 µg/ml), and thionine (BDH) (1.25 µg/ml). Sensitivity to antibiotics was measured using disks placed on plates streaked from 48-h-old cultures. The disks contained the following quantities of antibiotics: penicillin (5 U), streptomycin (100 µg), chloramphenicol (200 µg), aureomycin (250 µg), terramycin (250 µg), erythromycin (10 µg), tetracycline (250 µg), sulfonamide (1 mg), neomycin (250 µg), novobiocin (50 µg), oleandomycin (100 µg), nitrofurantoin (200 µg), and bacitracin (25 U). Bactrim Roche disks (Oxoid) containing 25 µg of trimethoprim-sulfamethoxazole and optochin disks (Oxoid) containing 25 µg of ethylhydrocupreine were tested similarly. Sensitivity to M and B 938 (4:4'-diamidinodiphenylamine dihydrochloride, May and Baker Co.) was determined by streaking cultures onto BM agar containing 12.5 mg/liter. Growth in the presence of potassium tellurite, sodium azide, thallous acetate, and sodium nitrite was tested on BM agar containing 320 mg/liter, 75 mg/liter, 500 mg/liter, and 5 g/liter, respectively. Growth in the presence of phenol was determined on BM agar containing 0.1, 0.2, 0.3, 0.4, or 0.5% (wt/vol) phenol.

The ability to grow at 5, 10, 15, 25, 30, 35, 37, and 44 C was determined on BM agar incubated for 10 days. Heat resistance was measured by adding 0.5 ml of bacterial suspension (a BM slant grown for 48 h washed off in 5 ml nutrient broth) to 4.5 ml of nutrient broth at 56 C. After intervals of 5, 10, 15 and 30 min, loopfuls were plated onto BM agar and incubated for 5 days.

Biochemical tests. Catalase activity was detected by the addition of "20 volume" hydrogen peroxide to 48-h-old BM agar cultures. Peroxidase activity was determined by the addition of reagent (0.5 crystalline benzidine hydrochloride dissolved in 3 ml of glacial acetic acid to which an equal volume of "20 volume" hydrogen peroxide had been added) to a BM agar culture and observing the development of a blue color. Oxidase was tested by the method of Kovács (44). BM

agar slopes containing 0.25% (wt/vol) cysteine hydrochloride were used for the detection of hydrogen sulfide; lead acetate paper strips suspended above the medium were used as indicator. Phosphatase was determined on BM agar containing 100 mg of phenolphthalein diphosphate per liter (added as 10 ml of a sterile 1% [wt/vol] aqueous solution to the autoclaved BM agar). After incubation for 5 days, the plates were inverted over strong ammonia solution and examined for a red color around the colonies. Gelatinase activity was determined on BM agar containing 0.4% (wt/vol) gelatin by flooding the plates, after 7 days incubation, with acid mercuric chloride solution. Hydrolysis of starch was determined on BM agar containing 0.5 soluble starch. After incubation for 7 days, some of the bacterial growth was scraped aside and the plates were flooded with Lugol iodine. Turbidity from egg yolk was determined on BM agar containing 10% (vol/vol) concentrated egg yolk emulsion (Oxoid) and an additional 1% (wt/vol) sodium chloride. Casein hydrolysis was recorded as clear zones on BM agar containing 20% (vol/vol) sterile skim milk. Esculin hydrolysis was determined on BM agar containing 0.05% ferric ammonium citrate and 0.5% esculin; after incubation the plates were examined for a brown coloration. Dextran or levan production was tested on BM agar plates containing 5% sucrose, which were incubated at optimum temperature; large mucoid colonies were taken to indicate dextran or levan production. Tetrazolium reduction was tested on BM agar plates adjusted to pH 6.0 and containing 1% (wt/vol) glucose and 2:3:5-triphenyl tetrazolium chloride (75 mg/liter) and examined after incubation for red-insoluble formazan. Production of ammonia was tested on BM agar plates containing 10 g of peptone/liter (L37, Oxoid) in place of nutrient broth no. 2 and casein hydrolysate. They were tested with Nessler reagent after incubation. Urease activity was tested by the micromethod of Lautrop (49). Arginine hydrolysis was detected using Thornley medium (80) modified as a micromethod. The medium was dispensed in 0.6-ml volumes in tubes (10 by 1.2 cm) and heavily inoculated. Development of a pink color was recorded after 2 and 24 h. Nitrate reduction was determined on BM agar plates containing 0.1% (wt/vol) KNO₃ and tested for nitrite by adding Griess-Ilosvay reagents. Deoxyribonuclease (DNase) activity was determined on the following medium: DNase agar (Oxoid) (39 g), Dowex I-X8 (5 g), distilled water (940 ml); mixture A was added after autoclaving. After incubation, plates were flooded with 1 M HCl and examined for clear zones. Production of hydrogen cyanide was determined on BM blood agar by use of sodium picrate indicator papers (68). Methyl red and Voges-Proskauer tests were determined in the following medium: brain-heart infusion (Oxoid) (37 g), glucose (5 g), K₂HPO₄ (5 g), distilled water (940 ml). After sterilization by autoclaving, mixture A was added, and the medium was dispensed in 30-ml bottles. After incubation, cultures were tested with methyl red indicator and O'Meara reagent.

Hydrolyses of xanthine and of tyrosine were determined by the method of Gordon and Smith (30)

using BM agar containing 0.5% (wt/vol) tyrosine or 0.4% (wt/vol) xanthine. Pigment production from tyrosine was also recorded. Phenylalanine deaminase activity was determined on BM agar containing 2 g of DL- β -phenylalanine per liter instead of the nutrient broth no. 2. Aqueous ferric chloride solution (10%, wt/vol) was used as the test reagent. Hydrolysis of Tweens 20, 40, 60, and 80 was determined on BM agar containing 1% (vol/vol) Tween. Precipitation around colonies was recorded as positive. Some strains produced a clear zone around growth on Tween 80, and this was also recorded. Production of acid from carbohydrates was determined on BM agar containing 0.2% (wt/vol) tryptone in place of nutrient broth no. 2, and only 0.1% (wt/vol) casein hydrolysate. The carbohydrates were prepared as 10% aqueous solutions and sterilized by filtration. After the basal medium was autoclaved, the carbohydrates were added to give a final concentration of 1%. Plates were incubated for 7 days, and acid production was detected by adding 2 drops of sterile 0.04% (wt/vol) aqueous bromothymol blue adjusted to pH 7.2. This technique was used to avoid inhibition of strains of *Bordetella pertussis* and some other strains by indicator incorporated in the medium. The following carbohydrates were tested: glucose, lactose, xylose, salicin, raffinose, α -methyl-D-glucoside, ribose, rhamnose, mannose, inositol, cellobiose, fructose, inulin, dextrin, dulcitol, L(+)-arabinose, adonitol, sorbitol, galactose, maltose, sucrose, mannitol, glycerol, and glucosamine hydrochloride. Glucose and lactose were also tested at 10% concentration. The mode of glucose metabolism (i.e. oxidative or fermentative) was determined by inoculating 1% glucose plates in duplicate and incubating one set aerobically and the other anaerobically in an anaerobic jar.

Computer methods. A numerical taxonomy program written at the Microbial Systematics Unit, Univ. of Leicester, England, was used. The majority of characters were two state and were coded 1 for positive, 0 for negative. Sixteen tests were scored as multistate characters, scoring 0 for negative and 1, 2, or 3 for increasing grades of positive response. Five of the 139 characters recorded were deleted from the data matrix because all strains gave identical results. Growth on 0.5% sodium chloride agar, growth at 30 C, and growth in the presence of optochin were positive for all strains, and no strain was capable of producing hydrogen cyanide or of fermenting dulcitol. The final $n \times t$ matrix contained 134 characters and 194 OTUs (strains). The limitations of the computer prevented all 194 OTUs from being sorted in a single run; this was overcome by selecting 89 OTUs with the specific epithets *pertussis*, *parapertussis*, and *bronchiseptica* and sorting these first (computation 1). Chosen representatives of the clusters that were found were then included with the remaining OTUs in the second run (computation 2). The similarity (s) coefficient employed, S_G , was that of Gower (32) using both positive and negative similarities. Some trials were made with the coefficient S_J , which excludes negative matches; the results were very similar and revealed no additional points of interest. Both single linkage and unweighted average linkage (UPGMA) clustering (75)

were used and gave very similar phenograms; results of the average linkage method are reported here. Vigor and pattern statistics (69) were also calculated.

RESULTS

Clustering of the strains. The results of two computations are presented as phenograms (Fig. 1 and 2). Figure 1 (computation 1) illustrates that all but three strains of *Bordetella* fell into three very tight clusters. Cluster 1 contained 30 strains received as *Bordetella pertussis*; cluster 2 contained 21 strains received as *Bordetella parapertussis*; cluster 3 contained 35 strains received as *Bordetella bronchiseptica*. Strain J70 (*Bordetella bronchiseptica* ATCC 15101) fell just outside cluster 3 but still retained an average similarity of over 91% with the rest of the cluster. Two aberrant strains, J44 and J67, are discussed later.

Figure 2 is a simplified phenogram showing the arrangements of all the strains in computation 2. These can be seen to form 13 clusters which are separated at the 66% similarity level into two large groups, A and B. Group A contains organisms of the genera *Haemophilus*, *Actinobacillus*, and *Pasteurella* that are discussed in a companion paper (72). Group B contains 150 strains, of which 134 fell into nine fairly well-separated clusters. The remaining 16 strains were satellites of one or another of these clusters.

Baumann, Doudoroff, and Stanier (8) have pointed out that, in the oxidase-negative moraxellas, glucose is metabolized by a nonspecific aldose dehydrogenase enzyme which can also oxidise D-galactose, D-mannose, D-ribose, D-xylose, L-arabinose, L-rhamnose, maltose, lactose, and cellobiose. Thus, production of acid from these sugars is highly correlated with the ability to produce acid from glucose. None of our strains was able to produce acid from other carbohydrates if it could not produce acid from glucose. Therefore carbohydrate acidification tests are not truly independent characters, and consequently their inclusion tends to increase the similarities between strains that attack no carbohydrates by increasing the number of negative matches.

For these reasons computation 2 was repeated after deleting 22 carbohydrate characters, retaining only the production of acid from 1% and 10% glucose and lactose, acid from 1% glucose anaerobically, and alkali from 1% lactose (computation 3). The simplified phenogram is shown in Fig. 3. There are relatively few differences from Fig. 2. The co-phenetic correlation coefficient for computation 3 was 0.81 (the correlation between the original

FIG. 1. Phenogram of strains of *Bordetella*, based on all 134 characters, Gower's Coefficient including negative matches, and UPGMA clustering. The co-phenetic correlation is 0.96. The similarity scale is directly comparable with that of Fig. 2, and is comparable with that of Fig. 3 if adjusted so that the new similarities are $(134S-22)/112$.

FIG. 2. Simplified phenogram of all strains based on all 134 characters, Gower's Coefficient including negative matches, and UPGMA clustering, redrawn to outline the major clusters. The co-phenetic correlation is 0.85. The apex of a triangle indicates the lowest similarity at which any branch arises among the strains in that cluster.

similarity values and those in the phenogram, ideally 1.0), showing that the phenogram is quite a satisfactory representation of the similarity matrix from which it was derived. In addition, the mean square distance from the line of perfect agreement in co-phenetic scatter diagrams (75) was calculated for each OTU. Those OTUs that showed highest values (and thus were OTUs whose relationships were most distorted by clustering) were J26, J128, and J172. Most of the other OTUs with rather high values were members of the *Moraxella-Neisseria*

clusters. High values would be expected from OTUs that were aberrant or intermediate between clusters; our general experience is that this is commonly so, although the trend is not very well shown here.

For several reasons we believe that Fig. 3 is a more accurate reflection of the taxonomy of these organisms than Fig. 2. A three-dimensional model based on average intergroup distances (using $1-S_G$ as a measure of distance) was in better agreement with Fig. 3 than Fig. 2 (R. Johnson, Ph.D. thesis, Univ. of England,

FIG. 3. Simplified phenogram of all strains from computation omitting 22 carbohydrate characters (computation 3). Conventions as in Fig. 2. The co-phenetic correlation is 0.81.

Leicester, 1972). In Fig. 3, three aberrant strains of *Neisseria* and *Moraxella* mentioned later (J150, J152, and J139), and also *Moraxella bovis* J130, appeared in group B together with other strains of these genera rather than in group A (as in Fig. 2). Also, the arrangement of clusters in Fig. 3 is in better accord with current views (50). We therefore base our conclusions principally on the results illustrated in Fig. 3, and the composition of the main clusters in that figure is given in Table 2.

Cluster B1 contained 44 strains of *Bordetella bronchiseptica* and *Alcaligenes* (mainly *A. faecalis*). Within B1 the strains of *B. bronchiseptica* formed a tight 95% phenon except for J70, which joined at 90% S. The strains of *Alcaligenes* did not form a similar tight cluster but were attached in ones, twos, or threes to

the main stem in the range 85 to 89% S. Strain J1 (*A. denitrificans*), however, joined the *bronchiseptica* phenon at 93% S.

Cluster B2 contained strains identified by us as *Acinetobacter lwoffii*. Although a type strain for this species is not available, the identification of these strains as *A. lwoffii* rests on the agreement with the original description (5), with results of other workers (8, 59, 60, 81), and with the inclusion in B2 of two strains, J27 and J29, believed to be authentic (8, 73).

Strains of this cluster were received under the names *Acinetobacter lwoffii*, *Moraxella lwoffii*, *Alcaligenes viscosus*, and *Alcaligenes metalcaligenes*, and just included in this cluster were strains received as *Mima polymorpha* var. *oxydans* and *Moraxella osloensis*.

Clusters B3 and B4 correspond to *Bordetella*

TABLE 2. *Strains of the group B clusters*

Cluster	No. in cluster	Strain no.	Designation of cluster
B1	44	J1-J8, J58-J66, J68-J72, J192-J209, J241-J244.	<i>Alcaligenes faecalis</i> and <i>Bordetella bronchiseptica</i>
B2	11	J9-J13, J27, J29, J132-J133, J136 ^a , J140 ^a .	<i>Acinetobacter lwoffii</i>
B3	30	J42-J43, J45-J53, J181, J186-J191, J229-J240.	<i>Bordetella pertussis</i>
B4	21	J54-J57, J211-J212, J214-J228.	<i>Bordetella parapertussis</i>
B6	14	J128-J131, J134, J137, J139, J143-J144, J146 ^a , J148-J150, J152.	<i>Moraxella</i> and <i>Neisseria</i> II
B7	3	J141-J142, J145.	<i>Moraxella</i> and <i>Neisseria</i> III
B8	7	J44, J154, J158-J159, J161-J162, J165.	<i>Brucella</i>
B9	13	J23-J24, J26, J30-J32, J34-J35, J37-J40, J67.	<i>Acinetobacter anitratus</i>
Unclustered	7	J25, J28, J33, J36, J41, J147 ^a , J210	

^a Cluster 5 (*Moraxella-Neisseria* I) was formed only in computation 2, and its four members, indicated by the footnote *a*, were redistributed in computation 3.

pertussis and *Bordetella parapertussis*, respectively. Within B3 there was no definite structure corresponding to antigenic phases, so that the phenetic changes associated with roughness were evidently slight.

Cluster B5 of Fig. 2 does not appear in Fig. 3, as its strains have been redistributed; strains J136 and J140 have moved into cluster B2, strain J146 has joined cluster 6, whereas J147 is now a satellite of B3 and B4.

Clusters B6 and B7 contained strains of several species of *Moraxella* and *Neisseria* but there was no differentiation of the two, which is not unexpected: these genera are close taxonomically, and the speciation within them is uncertain.

Cluster B8 contained four nomenspecies of *Brucella*, the three classical ones, *B. abortus*, *B. suis*, and *B. melitensis*, and also the organism from the dog, *B. canis*. They formed an 83% phenon; there were too few strains to show whether the internal arrangement corresponds with the nomenspecies. Though fewer characters were available for these strains, we believe this deficiency has not greatly biased the relationships involving *Brucella*.

Cluster B9 represented *Acinetobacter anitratus*. It also included strains received as

Achromobacter haemolysans (J26), *Achromobacter haemolyticus* (J31, J32), *Achromobacter conjunctivae* (J34), *Achromobacter mucosus* (J35), and *Achromobacter lacticus* (J37). The grouping of the last two agrees with previous work (8, 81), but there are some discrepancies regarding the first four strains (see Discussion).

Strains falling outside the clusters. Several unclustered strains require comment. J25 (*Acinetobacter calcoaceticus*) most closely resembled strains of cluster B9 at around 75% similarity. It differed from cluster B9 in being sensitive to 5% bile salts, in its dye sensitivities, and in being resistant to penicillin. It raises problems of nomenclature (see Discussion). *Achromobacter liquefaciens* ATCC 15716 (J36) did not share high similarities with any cluster. Thornley (81) examined this strain and found most of the cells to be gram positive. We confirmed this although several of the results obtained (particularly production of hydrogen sulfide, acid from various carbohydrates, and ammonia from peptone) differed from those obtained by Thornley. It probably does not belong to this area of bacteria. The results obtained for J41 (also called *Vibrio* O 1) agreed largely with those obtained by Fewson (26, 27). This strain shared high similarities with

cluster B3 but differed from these strains in being oxidase negative, lecithinase positive, tyrosinase positive, producing hydrogen sulfide, growing on nutrient agar, and in clearing Tween 80. Furthermore, it was resistant to penicillin but sensitive to sulfonamide.

Strain J44 (NCTC 8631) was received as *Bordetella pertussis* and at first seemed to be an aberrant strain of *Bordetella* (Fig. 1 and 2). In computation 3 it fell into B8 (*Brucella*). When tested by slide agglutination it gave a strong agglutination in *Brucella* antiserum but did not agglutinate in *Bordetella pertussis* antiserum. Bacon et al. (6) obtained a value of 57.5% for the G/C ratio of this strain, which is typical of other reported values for *Brucella*. Also, examination of crude protein extracts by polyacrylamide gel electrophoresis showed that J44 had a very similar protein pattern to J159 (a strain of *Brucella melitensis*). Other characters which it shared with strains of the *Brucella* cluster, but in which it differed from the strains of *B. pertussis*, included its resistance to 0/129 and Bactrim Roche and its production of urease. There seems little doubt that J44 belongs to *Brucella*, although it is somewhat atypical, and the National Collection of Type Cultures now lists it as *Brucella melitensis*.

We suspect that J139 became contaminated during study, as it was not saccharolytic on retesting. The ability of strain J130 to attack carbohydrates was, however, confirmed on checking. Its correct designation is still uncertain; it is clearly not a strain of *Moraxella bovis*.

A single strain of *Noguchia granulosis* (J153) was studied. It was an isolated member of group A. It was a gram-negative rod with a tendency to pleomorphism and grew on simple media but was inhibited by bile salts. Acid was not produced from lactose. It was nonmotile and catalase positive, and reduced nitrate. The oxidase reaction was weakly positive. It differed from the published descriptions of *Noguchia granulosis* (55, 56, 82) in not fermenting lactose, in motility, and in having a dull, nonviscid matte colony, and in growing well on nutrient agar and anaerobically. This raises doubts as to its authenticity. Finally, J172 (*Pasteurella haemolytica* T) has (Fig. 3) taken up a position with the other pasteurellas in group A.

Characters of the groups and clusters. Characters shown by all strains have been listed under Materials and Methods. All except three strains in group A could grow anaerobically and were fermentative, as they produced acid from glucose anaerobically. The three excep-

tions were J150 and J152 (*Neisseria pharyngis*) and J139 (*Moraxella bovis*). Only one (J180, *Francisella novicida*) of the 150 strains in group B was able to grow under strictly anaerobic conditions. However, some strains were able to grow anaerobically in the presence of nitrate, presumably because nitrate could act as an alternative electron acceptor to oxygen. Some strains of group B could produce acid from glucose aerobically but not anaerobically, so that group B strains are predominantly aerobic and either oxidative or nonsaccharolytic. Strains of group B also showed the following characteristics with few exceptions: they were gram negative, not markedly pleomorphic, nonpigmented (except cluster B4) did not require CO₂, haematin, or NAD for growth, were catalase and peroxidase positive, esculin, DNase, methyl red, and Voges-Proskauer negative, and produced ammonia from peptone. Characters most useful for differentiating the clusters of group B are shown in Table 3. In Table 4 are shown the percentages of positive results in the clusters from which the hypothetical median organisms or the cluster centroids can be obtained (51, 70).

In Table 4 the strains of *Alcaligenes* and *Bordetella bronchiseptica* have been tabulated separately.

Reproducibility of results. The effects of poor test reproducibility on taxonomic work have been discussed by Sneath and Johnson (71), who presented detailed findings from the present study. This was investigated by testing 21 strains in duplicate: the probability (*P*) of an erroneous result averaged 1.713%; if tests which gave identical results on both occasions in all 21 strains were excluded, the average value of *P* was 2.24%. These figures compare favorably with values of 4% or more found in other studies (31, 48, 51, 79). In effect it means that comparison of a strain against itself would result in a similarity of about 0.96 instead of the theoretical 1.0, which is satisfactory. The least reproducible tests were peroxidase, various morphological characters, and antibiotic sensitivities, hydrogen sulfide production, growth on bile salts, production of ammonia from peptone, and nitrate reduction.

Vigor and pattern statistics. The percentage difference between strains, i.e., the complement of similarity, can be partitioned into vigor and pattern difference, *D_V* and *D_P* (69). Vigor difference expresses the difference between strains in total metabolic activity as measured by the number of positive reactions. Pattern difference reflects which particular tests give positive results, and it attempts to make

TABLE 3. *Differential characters of clusters of group B^a*

Differential characters	<i>Brucella</i> B8 ^b	<i>Alcaligenes-Bord. bronchiseptica</i> B1	<i>Bordetella pertussis</i> B3	<i>B. parapertussis</i> B4	<i>Acinetobacter lwoffii</i> B2	<i>A. anitratus</i> B9	<i>Moraxella-Neisseria</i> II B6	<i>Moraxella-Neisseria</i> III B7
Pigment from tyrosine	-	-	-	+	-	-	-	+
Growth anaerobically with nitrate	+	-	-	-	-	-	-	-
Motility	-	+	-	-	-	-	-	-
Growth on 5% bile salts	v	+	-	+	+	+	-	+
Growth on 6% NaCl	-	v	-	-	-	-	-	+
Growth with M and B 938	NT	+	+	+	-	+	-	v
Sensitivity to penicillin	+	-	+	-	v	-	+	+
Oxidase	+	+	+	-	-	-	+	+
Urease	+	v	-	+	-	+	-	v
Nitrate reduction	+	+	-	-	-	-	v	v
Hydrolysis of starch	-	-	-	-	-	-	+	+
Hydrolysis of tyrosine	-	+	-	-	-	+	-	-
Clearing of Tween 80	-	+	-	-	v	-	-	-
Acid from 1% glucose aerobically	-	-	-	-	-	v	-	-
Acid from 10% glucose aerobically	+	-	-	-	-	v	-	-

^a Symbols: +, over 80% of strains positive within 7 days; -, less than 20% of strains positive within 7 days; v, 20 to 80% positive; NT, not tested.^b Cluster name and number.

TABLE 4. Characters of clusters of group B as percentage of positive reactions^a

Character	<i>Alcaligenes</i> B1 (7) ^b	<i>Bordetella</i> <i>bronchiseptica</i> B1 (37)	<i>Acinetobacter</i> <i>lwoffii</i> B2 (11)	<i>Bordetella</i> <i>pertussis</i> B3 (30)	<i>Bordetella</i> <i>parapertussis</i> B4 (21)	<i>Moraxella-Neisseria</i> II B6 (14)	<i>Moraxella-Neisseria</i> III B7 (3)	<i>Brucella</i> B8 (7)	<i>Acinetobacter</i> <i>anitratus</i> B9 (13)	<i>Agrobacterium</i> (2)	<i>Francisella</i> (2)
Gram reaction (2 days)	0	0	0	0	0	0	0	0	0	0	0
Retention of methyl violet (2 days)	0	0	9	0	0	79	100	0	0	0	50
Chains or filaments present (2 days)	0	0	18	7	0	14	33	0	31	0	0
Pleomorphism (2 days)	0	0	0	7	0	21	0	0	31	50	0
Cocci or coccobacilli (2 days)	71	97	100	97	0	79	100	100	92	0	50
Colony opaque	0	0	45	3	0	86	0	0	69	100	0
Colony viscous	0	0	36	0	0	14	0	0	0	0	0
Pellicle on broth	100	100	9	0	100	29	67	86	85	100	100
Diffusible brown pigment	0	0	0	0	100	14	0	14	0	0	0
Pigment from tyrosine	14	0	0	0	100	14	100	0	15	100 ^c	0
Dextran or levan production	0	0	0	0	0	0	0	NT	54	50	NT
Growth anaerobically	0	0	0	0	0	7	0	0	0	0	50
Growth anaerobically with nitrate	0	0	0	0	0	43	0	86	0	0	100
CO ₂ requirement	0	0	0	0	0	0	0	0	0	0	0
X factor requirement	0	0	0	0	0	0	0	0	0	0	0
V factor requirement	0	0	0	0	0	0	0	0	0	0	0
Growth on nutrient agar	100	100	100	7	100	86	33	100	100	100	100
Growth on blood agar	100	100	100	87	100	86	100	100	100	100	100
Hemolysis of horse red cells	0	0	0	73	0	29	0	0	0	0	0
Hemolysis of sheep red cells	0	3	9	3	95	29	0	NT	15	0	NT
Motility (14 days)	86	100	0	0	0	0	0	0	0	50	0
Growth on 5% bile salts	86	100	64	0	100	0	100	71	100	100	100
Growth on 10% bile salts	86	100	18	0	100	0	67	57	85	100	0
Growth on 40% bile salts	29	92	0	0	0	0	0	NT	31	0	0
Growth on 0% NaCl	100	100	100	100	100	29	100	100	100	100	100
Growth on 3% NaCl	100	100	91	7	100	86	100	71	100	100	100
Growth on 4.5% NaCl	86	100	82	0	10	71	100	0	100	50	0
Growth on 6% NaCl	71	100	0	0	0	0	100	0	8	0	0
Growth on 7.5% NaCl	43	0	0	0	0	0	100	0	0	0	0
Growth on 9% NaCl	0	0	0	0	0	0	33	0	0	0	0
Growth on 0.1% phenol agar	100	100	78	0	0	21	100	43	100	50	50

Growth on 0.2% phenol agar	43	0	56	0	0	0	7	0	14	46	0	50
Growth on 0.3% phenol agar	0	0	11	0	0	0	7	0	0	0	0	0
Growth on 0.4% phenol agar	0	0	0	0	0	0	7	0	0	0	0	0
Growth on 0.5% phenol agar	0	0	0	0	0	0	0	0	0	0	0	0
Growth on 320 mg of potassium tellurite per liter	100	0	0	0	0	0	7	0	NT	0	0	NT
Growth on 75 mg of sodium azide per liter	100	100	89	100	100	100	79	100	NT	100	100	NT
Growth on 0.5% thallous acetate	43	5	0	0	0	0	21	33	NT	15	0	NT
Growth on 12.5 mg of M and B 938 per liter	86	100	0	100	100	100	14	33	NT	92	50	NT
Growth on 0.5% sodium nitrite	100	100	100	100	100	100	79	67	NT	100	100	NT
Growth at 5 C (10 days)	0	0	0	0	0	0	0	33	NT	31	0	NT
Growth at 10 C (10 days)	71	70	55	0	0	0	0	67	NT	85	50	NT
Growth at 15 C (10 days)	100	100	100	37	100	100	57	100	86	100	100	100
Growth at 25 C (10 days)	100	100	100	100	100	100	10	100	100	100	100	100
Growth at 35 C (10 days)	100	100	89	100	100	100	100	100	100	92	100	100
Growth at 37 C (10 days)	86	100	78	100	100	100	100	100	100	92	50	100
Growth at 44 C (10 days)	14	27	22	0	0	0	21	33	NT	54	0	NT
Sensitivity to penicillin	0	0	67	100	0	0	100	100	71	8	0	0
Sensitivity to streptomycin	100	97	100	100	100	100	100	100	100	92	100	100
Sensitivity to chloramphenicol	100	100	100	100	100	100	100	100	100	100	100	100
Sensitivity to aureomycin	100	100	100	100	100	100	100	100	100	100	100	100
Sensitivity to terramycin	100	100	89	100	100	100	100	100	100	100	100	100
Sensitivity to erythromycin	86	97	100	100	100	100	100	100	100	92	100	100
Sensitivity to tetracycline	100	100	100	100	100	100	100	100	100	100	100	100
Sensitivity to sulfonamide	86	65	89	3	90	93	93	100	14	69	100	0
Sensitivity to neomycin	100	100	100	100	100	100	100	100	100	100	100	100
Sensitivity to novobiocin	100	100	89	100	100	100	100	100	71	92	100	100
Sensitivity to oleandomycin	86	95	100	100	100	100	100	100	57	92	100	100

TABLE 4.—Continued

Character	<i>Alcaligenes</i> B1 (7) ^b	<i>Bordetella</i> <i>bronchiseptica</i> B1 (37)	<i>Acinetobacter</i> Iwoffi B2 (11)	<i>Bordetella</i> <i>pertussis</i> B3 (30)	<i>Bordetella</i> <i>parapertussis</i> B4 (21)	<i>Moraxella-Neisseria</i> II B6 (14)	<i>Moraxella-Neisseria</i> III B7 (3)	<i>Brucella</i> B8 (7)	<i>Acinetobacter</i> anitrat- us B9 (13)	<i>Agrobacterium</i> (2)	<i>Francisella</i> (2)
Sensitivity to nitro- furantoin	43	49	44	100	100	100	100	100	38	100	50
Sensitivity to bacitracin	0	0	0	0	0	64	0	0	0	0	50
Sensitivity to Bactrim Roche	29	78	78	100	100	93	67	0	69	0	0
Sensitivity to 0/129	0	0	36	90	0	43	33	0	15	0	0
Sensitivity to thionine	0	0	36	0	0	43	33	0	8	0	0
Sensitivity to basic fuchsin	0	3	73	73	95	93	100	29	0	50	0
Sensitivity to methyl violet	0	30	100	83	33	100	100	57	62	100	100
Sensitivity to safranine	0	0	64	100	90	86	100	29	8	0	50
Sensitivity to pyronin	0	0	64	0	0	86	100	0	15	0	0
Heat resistance to 56 C, 5 min	100	100	100	100	100	64	100	NT	100	0	NT
Heat resistance to 56 C, 10 min	71	100	82	97	100	50	100	NT	92	0	NT
Heat resistance to 56 C, 15 min	71	100	64	97	100	36	100	NT	92	0	NT
Heat resistance to 56 C, 20 min	51	100	55	93	95	36	100	NT	92	0	NT
Catalase (2 days)	100	100	78	87	100	93	100	100	100	50	0
Peroxidase (2 days)	100	100	67	100	100	100	100	100	54	100	0
Oxidase (2 days)	100	100	22	100	0	100	100	86	8	100	0
H ₂ S production	71	0	89	43	0	86	33	57	54	100	50
Phosphatase (5 days)	14	0	0	0	0	14	0	0	8	100	50
Gelatin hydrolysis	29	0	0	0	0	50	0	NT	38	0	NT
Starch hydrolysis	0	0	22	0	0	100	100	0	0	0	0
Turbidity from egg yolk	0	0	33	0	0	43	67	0	69	0	0
Casein hydrolysis	0	0	0	0	0	14	0	0	31	0	0
Esculin hydrolysis	0	0	0	0	0	7	0	NT	8	100	0
Tetrazolium reduction	0	97	73	100	100	93	100	NT	31	100	NT
Ammonia from peptone	100	100	100	87	100	100	100	100	100	100	100 ^c
Urease (1 h)	43	97	27	0	100	0	67	100	69	100	0
Arginine hydrolysis (1 day)	100	100	82	100 ^c	100	86 ^c	100 ^c	100	100	100	0
Nitrate reduction	86	100	9	0	0	73	67	71	8	100	0

TABLE 4. -Continued

Character	<i>Alcaligenes</i> B1 (7) ^b	<i>Bordetella</i> <i>bronchiseptica</i> B1 (37)	<i>Acinetobac-</i> <i>ter lwoffii</i> B2 (11)	<i>Bordetella</i> <i>pertussis</i> B3 (30)	<i>Bordetella</i> <i>parapertus-</i> <i>sis</i> B4 (21)	<i>Moraxella-</i> <i>Neisseria</i> II B6 (14)	<i>Moraxella-</i> <i>Neisseria</i> III B7 (3)	<i>Brucella</i> B8 (7)	<i>Acinetobac-</i> <i>ter anitra-</i> <i>tus</i> B9 (13)	<i>Agrobacter-</i> <i>ium</i> (2)	<i>Francis-</i> <i>ella</i> (2)
Acid aerobically from L(+)-arabinose	0	0	0	0	0	0	0	NT	38	100	NT
Acid aerobically from dextrin	0	0	0	0	0	29	0	NT	31	0	NT
Acid aerobically from sorbitol	0	0	0	0	0	0	0	NT	0	0	NT
Acid aerobically from galactose	0	0	0	0	0	14	0	NT	69	100	NT
Acid aerobically from maltose	0	0	0	0	0	21	0	NT	62	0	NT
Acid aerobically from sucrose	0	0	0	0	0	29	0	NT	8	100	NT
Acid aerobically from mannitol	0	0	0	0	0	21	0	NT	0	0	NT
Acid aerobically from glycerol	0	0	0	0	0	14	0	NT	0	0	NT
Acid aerobically from glucosamine	0	0	0	0	0	29	0	NT	62	0	NT
Acid aerobically from inulin	0	0	0	0	0	0	0	NT	0	0	NT
Acid aerobically from adonitol	0	0	0	0	0	0	0	NT	0	50	NT

^a Symbols: figures indicate the percentage of strains positive within 7 days (unless otherwise noted).^b Cluster name and member. Number of strains indicated in parentheses.^c Weak but definite reactions.

allowance for factors such as growth rate, or length and temperature of incubation, that would otherwise contribute toward overall dissimilarity. D_P (or its complement S_P) can be clustered like other similarity coefficients and is intended to give taxonomic relationships that are less subject to disturbance by general factors of the kind mentioned.

The mean, range, and standard deviation of vigor of the clusters of group B are shown in Table 5, based on all characters including carbohydrate tests. The lowest values are shown by *Bordetella* and the highest by *Brucella*, but the spread is not great. In consequence of the fairly uniform values of vigor, its removal did not greatly alter the resemblances, so that clustering on D_P gave very similar results to Fig. 3. There were a few changes: the three clusters of *Moraxella* and *Neisseria* (B5, B6, and B7) were less distinct, so that these clusters appear to separate from each other in part because of vigor differences; some changes occurred in the composition of B2, and two strains of *Brucella* J159 and J161 were removed from cluster B8. It is worth noting that the four aberrant strains which appeared in group A in computation 2 had exceptionally high vigor (average 0.490). However, the pattern results did confirm that the difference between *Acinetobacter lwoffii* and *A. anitratus* was not simply accounted for by saying that *A. anitratus* is the metabolically active end of a spectrum containing both species, because the two clusters B2 and B9 remained separate in the D_P phenogram.

DISCUSSION

The results of the study reported here throw doubt on the taxonomic validity of the family *Brucellaceae* as given at present in *Bergey's Manual* (13). Eight genera were listed there: *Pasteurella*, *Bordetella*, *Brucella*, *Haemophilus*, *Actinobacillus*, *Calymmatobacterium*, *Morax-*

ella, and *Noguchia*. These have varied properties: some have a fermentative metabolism, others are oxidative, others do not attack carbohydrates. The range of G/C ratio, 33 to 70% (36), is also evidence of heterogeneity. There is already much evidence (41) to support two changes that are gaining increasing acceptance. (i) The plague bacillus and its allies should be segregated from the other pasteurellas as a genus, *Yersinia*, that appears close to *Enterobacteriaceae*, and they may be best included in that family. (ii) The genus *Moraxella* is very closely allied to at least some species of *Neisseria*, so that it might be best transferred to *Neisseriaceae* (50), and *Acinetobacter* is best temporarily associated with that family for practical reasons.

Our findings, as well as providing support for (ii), also suggest another major change: (iii) the fermentative genera of group A, *Actinobacillus*, *Pasteurella*, and *Haemophilus*, should be separated from the others. The more detailed evidence for the third point is discussed elsewhere (72), but we may note here that these three genera are phenetically very similar, and their G/C base ratios fall in the narrow range of 35 to 45% (36).

Although many neisserias and moraxellas are extremely similar phenetically, as noted in several reports as well as our own (7, 35, 81), the present study affords no assistance on whether *Moraxella* and *Neisseria* should be united, because we did not include *Neisseria gonorrhoeae*, the type species of the latter genus. There is some evidence that *Neisseria* is notably heterogeneous (41), so the question may instead be whether species such as *N. catarrhalis*, *N. caviae*, and *N. ovis* should be transferred to *Moraxella*.

It is not yet clear whether *Acinetobacter* should be included in the *Neisseriaceae*. *Acinetobacter* species grow well on ordinary media and remain viable without subculture for quite long periods, whereas both *Moraxella* and

TABLE 5. Vigor values of the clusters of group B

Cluster	Range	Mean	SD
B1 <i>Alcaligenes faecalis</i> - <i>Bordetella bronchiseptica</i>	0.353-0.424	0.390	0.020
B2 <i>Acinetobacter lwoffii</i>	0.325-0.406	0.374	0.023
B3 <i>Bordetella pertussis</i>	0.317-0.344	0.330	0.011
B4 <i>Bordetella parapertussis</i>	0.386-0.405	0.398	0.005
B6 <i>Moraxella</i> - <i>Neisseria</i> II	0.328-0.581	0.436	0.060
B7 <i>Moraxella</i> - <i>Neisseria</i> III	0.426-0.496	0.465	0.036
B8 <i>Brucella</i>	0.357-0.519	0.466	0.052
B9 <i>Acinetobacter anitratus</i>	0.408-0.504	0.454	0.037

Neisseria are much more fastidious and sensitive to aging. The G/C base ratios are close to those of *Moraxella* and *Neisseria*, but Johnson, Anderson, and Ordal (40) found little evidence of nucleic acid hybridization between oxidase-positive moraxellas and oxidase-negative acinetobacters. They reached conclusions similar to Stenzel and Mannheim (77) and Baumann, Doudoroff, and Stanier (7) that the two should be kept separate. Recent genetic studies (42) support this. The reliability of the oxidase reaction for separating *Moraxella* and *Acinetobacter* is questioned by Juni (42) and also by Thornley (81), who found that many of her phenon 4 strains (equivalent to *Acinetobacter lwoffii*) were oxidase positive. Two out of nine strains of *A. lwoffii* studied by us were also oxidase positive, so it would seem that splitting the moraxellas and acinetobacters on the oxidase reaction may not be entirely satisfactory.

We recognize two species of *Acinetobacter*, *A. lwoffii* and *A. anitratus*, equivalent to clusters B2 and B9, respectively. They are well defined and reasonably distinct on both similarity and pattern coefficients. This is in agreement with results of previous studies (8, 59, 60, 81). The pattern results show that the difference between these species is not simply one of metabolic activity. Nevertheless, transformation can occur between them (42), they are both rather diffuse groups, and it is possible that they overlap to a small extent, or that occasional intermediates occur. Strains of *A. anitratus* are almost always saccharolytic, and it is generally thought that *A. lwoffii* is nonsaccharolytic. Baumann, Doudoroff, and Stanier (8) found a few saccharolytic strains of the *A. lwoffii* type. Three of these were also studied by us (J26, J32, and J34), and although we found them to be nonsaccharolytic, evidence has recently been published (74) that some strains of *A. lwoffii* can produce acid from glucose in the ammonium salt sugar medium of Smith, Gordon, and Clark (67), although Henderson (34) had found that none of five strains of *A. lwoffii* he examined could do so on this medium. Using a modification of this medium (Johnson, unpublished data), in which bromothymol blue indicator was used to replace the less sensitive bromocresol purple, it was found that most strains of *A. lwoffii* will produce definite acid from glucose, xylose, arabinose, and ethanol; strains of *A. anitratus* produce acid from these sugars and, in addition, cellobiose, rhamnose, and lactose. Thus, even on this medium the two species can be separated, although *A. lwoffii* cannot now be

regarded as strictly nonsaccharolytic. Baumann, Doudoroff, and Stanier (8) also had some strains similar to *A. anitratus* that were nonsaccharolytic; they formed a distinct group, however, and the only strain of this kind that we also studied, J41, did not fall into any cluster. There may be, therefore, at least one additional species of *Acinetobacter* of which we had only a single representative. The position is, however, not entirely clear, because a few strains that in the present study fell into cluster B9 (J26, J31, J32, J34) were found by Baumann, Doudoroff, and Stanier to fall outside their group A (which contained *A. anitratum* and *A. calcoaceticus*).

Further evidence that other species may exist is provided by *Acinetobacter calcoaceticus* (J25). The Subcommittee on the Taxonomy of *Moraxella* and Allied Bacteria (50) suggests that this name should be the correct name for *A. anitratus*. We found that it was not a typical strain of *A. anitratus*, as it did not enter cluster B9 in either computation 2 or 3, nor using pattern coefficients (showing that the atypicality is not due simply to loss variation). Neither was it typical of *A. anitratus* in the study of Baumann, Doudoroff, and Stanier (8). It may therefore be premature to regard these two as synonyms.

Acinetobacter lwoffii may be phenetically intermediate between *Moraxella* and *Bordetella*; indeed Steel and Cowan (76) placed *B. parapertussis* in the genus *Acinetobacter*. There are difficulties in distinguishing between *B. parapertussis* and *A. lwoffii*, particularly if the strains of the latter produce an alkaline reaction in the Hugh and Leifson oxidation-fermentation medium. The high phenetic similarity between them is shown by an average intergroup similarity of 80.2%. Only two clearly differential characters were found, the ability of *B. parapertussis* to produce a brown, diffusible pigment from tyrosine and to grow in the presence of the antibacterial compound M and B 938. However, G/C base ratios were determined on six strains of *B. parapertussis* (Johnson, Ph.D. thesis, Univ. of Leicester England, 1972), and these varied from 67.7 to 70.0%; this difference of about 25% from published values for *A. lwoffii* suggests that they should not be included within the same genus. The ratios found by Johnson for six strains of *B. pertussis* were almost the same, ranging from 67.0 to 69.8%.

Another problem is the relationship between *Alcaligenes* and *Bordetella bronchiseptica*. In this study all strains of *A. faecalis*, one strain of *A. riscosus* and strains of *A. denitrificans* and

A. odorans fell into the same cluster as *B. bronchiseptica*, with the strains of *Alcaligenes* having a higher average similarity to the strains of *B. bronchiseptica* than to each other. The only characters that differentiated them were tetrazolium reduction and growth on potassium tellurite agar (Table 4). The species of *Alcaligenes* included in this group are those which are motile and oxidase positive. This distinguishes them from the nonmotile, oxidase-negative strains which we believe are misidentified strains of *Acinetobacter lwoffii*.

There has been much confusion over the genus *Alcaligenes* and its type species, *A. faecalis*, the *Bacillus faecalis alcaligenes* of Petruschky (57). The original description of the genus by Castellani and Chalmers (16) was not very detailed. Petruschky's original description could apply equally well to *B. bronchiseptica* if the source of isolation was ignored. Hugh (Ph.D. thesis, Loyola University, Chicago, 1953) united peritrichous, nonsaccharolytic, gram-negative bacteria (such as *Bordetella bronchiseptica*, *Alcaligenes faecalis*, and *A. denitrificans*) to form his group I, and he agreed with an earlier report by Conn (17) that Petruschky's description was too vague to reidentify the organism. Many workers have found that urease production is the most useful test for differentiating *A. faecalis* from *B. bronchiseptica*, but in our study three out of seven strains of *Alcaligenes* were urease positive. Recently Pickett and Pedersen (58) found differences in the production of alkali from certain amides and organic salts, and these may prove useful after further evaluation. There is abundant evidence for retaining *B. bronchiseptica* in the same genus as *B. pertussis* and *B. paraptussis* on physiological, biochemical, serological, and genetic grounds (20, 28, 46, 64, 66). The question is, therefore, whether *Alcaligenes* should be united with *Bordetella*. The generic name would be *Alcaligenes* for reason of priority. However, the genus-specific antigens of *Bordetella* and the species-specific antigens of *B. bronchiseptica* (4, 21, 47) have not been identified in *A. faecalis*. For the present it seems wiser to keep the two genera separate.

The similarity between *Bordetella* and *Brucella* has been known for many years (10, 83). However, the genera also differ sharply in several ways: *Brucella* metabolizes alanine and DL-asparagine initially (29, 52), whereas glutamic acid and proline are the preferred amino acids for *Bordetella* (65). There have not been any reports of antigens shared by the two genera, and Hoyer and McCullough (38) found in deoxyribonucleic acid pairing studies that

there was no detectable pairing between *Brucella* and *Bordetella bronchiseptica*.

The positions of two genera, *Calymmatobacterium* and *Noguchia*, still remain in doubt. We were unable to obtain any strains of *Calymmatobacterium*, and we have doubts on the authenticity of the only strain of *Noguchia granulosis* available.

There remain two problems of nomenclature. It is not clear who first legitimately published the specific epithet *pertussis* for the whooping-cough bacillus. Some have attributed the name to Bordet and Gengou (11), who originally isolated the organism, but they never used this specific epithet in any of their publications. Breed, Murray, and Smith (13) attributed the name to Holland (37), but this is incorrect because names listed by Holland are not validly published (39, Rule 12c). Buchanan, Holt, and Lessel (14) give two names for this organism which they state were validly published and are legitimate. The first was *Bacillus tussis-convulsivae* Affanassjew 1887. Affanassjew did not, however, isolate the whooping-cough bacillus. Koplik (43) isolated an organism which he considered identical to the one described by Affanassjew, yet it was motile and grew anaerobically. These are not properties of *B. pertussis*. Migula (53) described *Bacillus pertussis*, which he attributed to Affanassjew, but Migula's organism was able to grow on gelatin and also grew anaerobically. Clearly *Bacillus tussis-convulsivae* cannot be identified with the whooping-cough bacillus. The other name cited by Buchanan, Holt, and Lessel is *Haemophilus pertussis* Pribram 1933. Pribram mentions *H. pertussis* only in the list of strains maintained in the Vienna Microbiological Collection; the organism is attributed to Bordet 1906 and not, as it should have been, to Bordet and Gengou (11), and therefore it is not validly published (39, Rule 12c) and also lacks a description or a reference to an effectively published description. Ferry and Noble (25) used the name *Bacterium pertussis* for the whooping-cough bacillus. They did not attribute the organism to Bordet and Genou (11), neither did they indicate they were naming or renaming a bacterial species, so the validity of publication is dubious. Bergey et al. (9) published an avowed taxonomic description of an organism, *Haemophilus pertussis* (Bordet and Gengou) Committee, Society of American Bacteriologists, which is clearly the whooping-cough bacillus. Although they incorrectly attribute the epithet to Bordet and Gengou, this name appears undoubtedly valid and has priority over that of Pribram. We therefore take the view

that the correct name for the whooping-cough bacillus is *Bordetella pertussis* (Bergey et al. 1923) Moreno-López 1952.

The second problem is the correct name for *Bordetella bronchiseptica*. This organism was originally isolated from dogs by Ferry (22) and named *Bacillus bronchicanis* (23). In a later paper, Ferry (24) changed the specific epithet to *bronchiseptica* because the organism was able to produce disease in other animals besides dogs. Under Rule 23 of the International Code of Nomenclature of Bacteria (39), a name cannot be changed because it is inappropriate, so that the correct epithet remains *bronchicanis*. This epithet has been used by few authors since, except Haupt (33). The epithet *bronchiseptica* is almost universally used; to change it back to *bronchicanis* would cause much confusion, and it would seem better to conserve the well-known one.

The taxonomy of the *Brucellaceae* would, in our opinion, be improved by reducing the genera to three, *Brucella*, *Bordetella* and *Alcaligenes*. The fermentative genera *Haemophilus*, *Pasteurella*, and *Actinobacillus* should be removed and could form the basis of a separate family. The three genera that would remain are phenotypically very similar. They have a marked preference for metabolizing amino acids (29, 52, 66). Two genera show a requirement for accessory growth factors: *Bordetella* requires nicotinamide and *Brucella* requires thiamine. Morphologically, they are all aerobic, gram-negative coccobacilli that show little tendency towards pleomorphism. They are usually oxidase positive, although *Bordetella parapertussis* is an exception. Finally, all have high G/C base ratios, usually above 55%. The position of *Francisella* is uncertain and requires further study.

The members of *Brucellaceae* have the reputation of being difficult to study, but we believe they are not particularly difficult provided the bacteriologist spends some time devising suitable culture media and standardizing test conditions. There is still a need to develop new biochemical tests. *Bordetella*, *Alcaligenes*, *Brucella*, and *Acinetobacter* give a large number of negative results in currently used identification tests, so the development of new tests, and perhaps the inclusion of carbon and nitrogen utilizations, should prove valuable for future work.

ACKNOWLEDGMENTS

The work reported was done during the tenure by one of us (R.J.) of a Scholarship from the Medical

Research Council of Great Britain. The assistance of M. J. Sackin with computing is also gratefully acknowledged.

REPRINT REQUESTS

Address requests for reprints to: Dr. P.H.A. Sneath, Medical Research Council, Microbial Systematics Unit, University of Leicester, University Road, Leicester, England.

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