IDENTIFICATION OF *BACILLUS ANTHRACIS* BY API TESTS

N. A. LOGAN*, J. A. CARMAN†, J. MELLING† AND R. C. W. BERKELEY

*Department of Microbiology, The Medical School, University Walk, Bristol BS8 1TD,
and †Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire
SP4 0JG*

SUMMARY. API and morphological tests were examined for their ability to distinguish between 37 *Bacillus anthracis* strains (virulent and avirulent) and 194 strains of closely related *Bacillus* species (*B. cereus*, *B. mycoides* and *B. thuringiensis*). In addition, 34 strains of *B. anthracis* and four of *B. cereus* were tested by several other methods that included capsule formation, ability to grow on a selective medium, and sensitivity to phage. It was found that virulent strains of *B. anthracis* were easily separated from the closely related *Bacillus* species by most of the test methods; but separation of slightly virulent and avirulent strains of *B. anthracis* from the closely related species could be done only by API and phage-sensitivity tests.

INTRODUCTION

The recognition of typical virulent strains of *Bacillus anthracis* isolated from clinical material is a comparatively simple problem (Sterne, 1967), but the identification of strains from non-clinical material may not be so easy (Burdon, 1956; Biegeleissen *et al.*, 1962; Knisely, 1965; Wilson and Miles, 1975; Clarke *et al.*, 1977). Furthermore, the possibility that atypical, slightly virulent or avirulent strains of *B. anthracis* may be present in a specimen should always be borne in mind.

Because of the considerable similarity between *B. anthracis* and *B. cereus*, many techniques for the selective isolation (Pearce and Powell, 1951; Morris, 1955; Green and Jamieson, 1958; Knisely, 1965 and 1966) and identification (Brown and Cherry, 1955; Chadwick, 1959 and 1963; Bonventre and Eckert, 1963) of *B. anthracis* have been developed, but none is absolutely reliable. *B. cereus*, although often regarded as a laboratory contaminant, is now recognised as a pathogen and isolated as such with increasing frequency (Turnbull *et al.*, 1979; Gilbert *et al.*, 1981).

It is not usually difficult to separate virulent strains of *B. anthracis* from *B. cereus*, but the relationship between avirulent *B. anthracis* strains and *B. cereus*, about which there has been argument for many years, remains unclear.

In this paper, we show that avirulent and slightly virulent strains of *B. anthracis*

Received 28 Sep. 1984; accepted 26 Nov. 1984.

* Present address: Department of Biological Sciences, Glasgow College of Technology, Cowcaddens Road, Glasgow G4 0BA.

give the biochemical reactions of typical *B. anthracis* strains in API tests and are easily separated from other members of the *B. cereus* group on this basis.

MATERIALS AND METHODS

Organisms. The 37 *B. anthracis* strains studied are listed in table I. *B. cereus* strains were isolated from soil (4 strains), Pitch Lake, Trinidad (1), food and milk (2), uncooked rice (7), custard pie (1), food poisoning outbreaks, commonly diarrhoeal types (10), brain abscess (1), artificial hip joints (2), burn (1), blood (2), wounds (8), other infections (22), bovine source (1), pig (1), rat (1), anticorrosion coating (1), unspecified sources (12); one was the *B. cereus* "type strain" and others were received as *B. albolactis* (4), *B. anthracoides* (1), and *B. fluorescens* (3). Thirty further strains of *B. cereus*, including those isolated from outbreaks of emetic-type food poisoning (see Taylor and Gilbert, 1975; Gilbert and Parry, 1977), belonged to serotypes 1 (14 strains), 3 (6), 5 (4), 8 (5) and 12 (1); 11 of these strains had been demonstrated to possess emetic activity (Logan *et al.*, 1979). Four additional strains of *B. cereus* were freshly isolated from soil from Gruinard Island, which was contaminated with spores of the type strain of *B. anthracis* in the 1940s (see Manchee *et al.*, 1981). *B. mycoides* strains were isolated from soil (5 strains), dust (1), sheep faeces (1), grass (1), milk (1), cheese (1), and unspecified sources (12); a further three were received as *B. praussnitzii*. Fifty-three *B. thuringiensis* strains were of serotypes 1 (4 strains), 2 (5), 3a (5), 3ab (3), 4ab (6), 4ac (1), 5ab (2), 6 (4), 7 (2), 8 (1), 9 (2), 10 (2), 11 (1), 12 (1), 13 (1), 14 (4), and unknown (9).

Spore suspensions for virulence tests. Strains were grown on Nutrient Agar (Oxoid), containing $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 5 mg/L to promote rapid sporulation (Claus and Schaab-Engels, 1977) in Roux bottles for 48 h at 37°C. Sporulation was monitored by the microscopical examination of films. Spores were washed off the medium with water, heated at 60°C for 90 min, and viable counts were made. Viable counts were accurate to within $\pm 10\%$; and in any one set of dilutions for inoculation, the ten-fold differences between dilutions were accurate to within $\pm 5\%$. Dilutions, made in water because saline caused aggregation of spores, gave final counts in the range from 8 to 10^6 spores in 0.1 ml.

Virulence tests. Mice weighing 18–22 g were distributed in groups of ten, five to a cage. Each mouse was given 0.1 ml of a spore suspension intraperitoneally. Each set of tests included 10 mice given 10^3 spores of strain BO463 (Vollum; type strain) as the positive control. Mice were held for 6 days, inspected frequently, and deaths counted each day. There was no evidence that the few incidents of cannibalism affected the results.

Morphological tests. Strains of all species except *B. anthracis* were grown overnight at 30°C on plates of Nutrient Agar (Difco) containing $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ 5 mg/L and examined in saline suspension smears by phase contrast microscopy at $\times 1000$ magnification for the presence of chains of cells, vacuoles, and motility. Cell widths were measured with an AEI Image-Splitting Eyepiece (Vickers Instruments, Haxby Road, York); this is more accurate than an eyepiece graticule (Quesnel, 1971; Logan, 1980; Logan and Berkeley, 1981). Lack of motility was confirmed by observing cells in water of condensation taken from nutrient-agar slopes incubated at 30°C for 6 h or until growth appeared thereafter. For the observation of sporangial morphology, plates were reincubated until spores appeared. Smears were examined for spore shapes and positions, swelling of sporangia, and the presence of crystalline inclusions (see Logan, 1980).

B. anthracis strains were grown on Brain Heart Infusion Agar (Oxoid) in Roux bottles overnight at 30°C in sealed transfer containers and growth was washed off with sterile normal saline. The washings were used for the preparation of API inocula and for morphological studies. For the latter purpose dense suspensions in normal saline were treated with formaldehyde 1% v/v overnight so that examinations could be made on killed organisms without the use of the safety cabinets in which all studies with live *B. anthracis* strains were made. Less than 5% of the organisms had sporulated. This was sufficient for the observations on sporangia to be made but not enough to affect adversely the use of the API strip inocula. Motility was detected as diffuse growth around the line of stab inoculation in tubes of motility medium (Cowan, 1974). Single strains of *B. cereus*, *B. mycoides* and *B. thuringiensis* were used as controls for all studies on *B. anthracis*.

API tests. *B. cereus*, *B. mycoides* and *B. thuringiensis* were grown as lawns on plates of Nutrient Agar overnight at 30°C. *B. anthracis* and control strains of *B. cereus*, *B. mycoides* and *B. thuringiensis* were grown on Brain Heart Infusion Agar in Roux bottles overnight at 30°C. Cells were harvested in sterile normal saline and suspensions prepared, to the opacity of McFarland's (1907) standard tube no. 3 (15 International Opacity Units, OD 0.65 at 450 nm), in 5-ml volumes of saline and in 10-ml volumes of API 50CHE medium (API Laboratory Products Ltd, Grafton Way, Basingstoke, Hampshire) for the API 20E strip and the API 50CH gallery respectively. The first 12 tests of the 20E strip and all the tubes of the 50CH gallery were inoculated according to the manufacturer's instructions, but the tubes of the 50CH gallery were not sealed with mineral oil. Strips and galleries were incubated at 30°C, and readings were made at 24 and 48 h. Reagents for the tryptophan deaminase, indole, VP, and nitrate reduction tests of the API 20E strip were added at the time of the second reading. Roux bottles and API strips and galleries containing *B. anthracis* and control strains were incubated in sealed transfer containers for safety.

Results were scored from 0 (negative) to 5 (strong positive) for computer analyses as described by Logan (1980) and Logan and Berkeley (1981). For the preparation of tables of percent positive results, scores of 3 and above were regarded as positive.

Details of the development of an API identification scheme for *Bacillus* species are given by Logan (1980), Logan and Berkeley (1981) and Berkeley *et al.* (1984).

Bicarbonate agar test. Strains were grown on plates of Nutrient Agar (Oxoid) containing sodium bicarbonate 0.5% w/v and horse serum 5% in an atmosphere containing CO₂ c.60% at 37°C for 24 h. After incubation they were examined for mucoid growth.

M'Fadyean reaction. Strains were incubated in defibrinated horse blood for 8 h at 37°C. Smears were then made, gently heat-fixed, stained with polychrome methylene blue (Gurr, BDH Chemicals Ltd, Broom Road, Poole, Dorset) and examined at ×1000 magnification for blue rods surrounded by capsular material stained purplish pink.

Colonial texture test. Colonies on plates of Nutrient Agar (Oxoid) incubated at 37°C for 24 h were teased with an inoculating loop and observed for the characteristic tenacity of *B. anthracis* colonies or the butyrous texture of *B. cereus* colonies.

Growth on the selective medium of Knisely (1966). Strains were inoculated on to plates of PLET agar (Heart Infusion Agar, Difco, containing polymyxin 30 units/ml, lysozyme 40 µg/ml, disodium ethylenediaminetetraacetate 300 µg/ml and thallous acetate 40 µg/ml) and incubated at 37°C for 24 h. This medium allows the recovery of *B. anthracis* strains but inhibits the growth of most *B. cereus* strains and most other *Bacillus* species.

Sensitivity to phage. Lawns of the strains were prepared on plates of Nutrient Agar (Oxoid) by spread inoculating with 0.1 ml of an overnight broth culture. One loopful of γ-phage (Brown and Cherry, 1955) suspension containing 10⁷ plaque-forming units/ml was placed in the centre of the plate. Inocula were allowed to dry and plates were incubated at 37°C for 18 h and examined for areas devoid of growth.

RESULTS

Virulence tests

Table II shows that the *B. anthracis* strains were broadly divisible into three groups—virulent, slightly virulent and avirulent. Where the histories of the strains were known, the results were as expected; thus the type strain (Vollum B0463) showed high virulence, the Sterne “avirulent” strain (B0464) low virulence, the two “teaching” or “class” strains (B0474, B0475) of the late Dr T. Gibson, low virulence; eleven recent isolates from anthrax outbreaks (B0477–B0482 and B1121–B1125) showed high virulence and three strains (B1117–B1119), received in response to a request for avirulent strains, low virulence. Five strains (B1126–B1128, B1130 and B1131) which were maintained on slopes between the 1920s and the 1940s (R. E. Gordon personal communication) comprised two avirulent and three virulent strains.

TABLE I
Strains of Bacillus anthracis

Strain no.	Source and collection no.	Origin or Comment
B0463	NCTC 10340	Vollum; from cow; type strain
B0464	NCTC 8234	Spears, 34 F 2 (Sterne); avirulent
B0467	NCTC 2620	Hankow; Chinese hide; Robertson, 1928
B0468	NCTC 5444	St Mary's; Fleming, 1938
B0469	NCTC 1328	Fildes, 1922
B0470	NCTC 109	Paddington VI; shaving brush, 1920
B0471	Gibson 958	Armitage
B0472	Gibson 961	NCTC 2620
B0473	Gibson 962	Lister 4991
B0474	Gibson 1010	"Class" strain
B0475	Gibson 1011	Soil culture of G1010
B0476	Gibson 1012	Cheyne
B0477	Wbrg A52-77	Cattle
B0478	Wbrg A73-77	Groundnut meal
B0479	Wbrg A183-78	Groundnut meal
B0480	Wbrg A187-78	Cow, Devon
B0481	Wbrg A188-78	Bovine source, Devon
B0482	Wbrg A191-78	Dairy cow, Shropshire
B0483	CAMR B1	Avirulent
B0484	CAMR M36	Enhanced virulence for rabbits
B1115	CAMR M84	Soil, Taunton
B1116	Wbrg A219-78	Cow
B1117	Böhm 1	Sterne strain
B1118	Böhm 2	NP1/R protective antigen production
B1119	Böhm 15	Vaccine strain, Roumania
B1120	Glfd C11	...
B1121	Glfd C73	Cerebro-spinal fluid, fatal case, 1974
B1122	Glfd C111	Blood, fatal case, 1974, penicillin resistant
B1123	Glfd C149	Skin lesion, 1976, from wool
B1124	Glfd C164	Skin lesion, 1976, from hides
B1125	Glfd C165	Skin lesion, 1976, infected animal
B1126	ATCC 240	Jordan, NIH (NRS 10081)
B1127	ATCC 937	Eichorn 1 (NRS 1010)
B1128	ATCC 938	Eichorn 2 (NRS 1011)
B1129	ATCC 10	NIH, 152 (NRS 1007)
B1130	ATCC 241	Jordan, NIH (NRS 1009)
B1131	ATCC 944	Levine, 2025; creamery waste

Abbreviations and designations: ATCC, American Type Culture Collection strains from Dr R. E. Gordon, Rutgers University, New Brunswick, NJ, USA; Böhm, Dr R. Böhm, Universität Hohenheim, Stuttgart, West Germany; CAMR, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire; Eichorn, A. Eichorn, Lederle Laboratories, Pearl River, New York, USA; Gibson, from collection of the late Dr T. Gibson now held at the University of Bristol; Glfd, Dr J. R. Davies, Public Health Laboratory, Guildford, Surrey; Jordan, E. O. Jordan, University of Chicago, Chicago, IL, USA; Levine, M. Levine, Iowa State University, Ames, IO, USA; NCTC, National Collection of Type Cultures, Central Public Health Laboratory, Colindale, London; NIH, National Institutes of Health, Bethesda, MD, USA; NRS, N. R. Smith Collection, US Department of Agriculture, Washington DC, USA; Wbrg, Dr D. Spackman, Central Veterinary Laboratory, Weybridge, Surrey.

Morphological and API tests

The results of morphological and API tests for all the species studied, those for *B. anthracis* classified according to virulence, are summarised in table III. The different species or groups showed close similarity. No test was specific for *B. anthracis*, and in only four tests—arginine dihydrolase (ADH), growth on Simmons' citrate medium and production of acid from glycerol and cellobiose—did *B. anthracis* alone give consistently negative results.

TABLE II
Results of tests for virulence of 34 *B. anthracis* strains in mice

Strain no.	Total deaths in groups of 10 mice (days to reach total) receiving the following doses of spores								Virulence
	8	40	100	200	10 ³	10 ⁴	10 ⁵	10 ⁶	
B0463	0 (6)	4 (3)	...	8 (5)	10 (4)	...	...	...	V
B0464	...	...	...	...	0 (6)	2 (6)	3 (4)	6 (4)	LV
B0467	...	...	1 (4)	...	9 (5)	10 (3)	10 (4)	...	V
B0469	...	...	0 (6)	...	0 (6)	0 (6)	0 (6)	...	AV
B0470	...	...	0 (6)	...	0 (6)	0 (6)	0 (6)	...	AV
B0471	...	...	5 (4)	...	7 (4)	10 (5)	10 (3)	...	V
B0472	...	...	1 (3)	...	6 (5)	10 (4)	10 (3)	...	V
B0473	...	...	7 (5)	...	7 (4)	10 (4)	10 (4)	...	V
B0474	...	...	0 (6)	...	0 (6)	0 (6)	2 (5)	...	LV
B0475	...	...	0 (6)	...	0 (6)	1 (4)	1 (6)	...	LV
B0476	...	...	4 (4)	...	10 (5)	10 (3)	10 (3)	...	V
B0477	...	...	5 (3)	...	9 (4)	10 (3)	10 (2)	...	V
B0478	...	...	8 (4)	...	10 (3)	9 (4)	10 (2)	...	V
B0479	...	...	7 (4)	...	10 (4)	10 (2)	10 (2)	...	V
B0480	...	...	9 (4)	...	10 (3)	10 (2)	10 (2)	...	V
B0481	...	...	8 (6)	...	10 (4)	10 (2)	10 (2)	...	V
B0482	...	...	8 (6)	...	10 (3)	10 (2)	10 (2)	...	V
B0483	...	...	0 (6)	...	0 (6)	0 (6)	0 (6)	...	AV
B0484	1 (5)	1 (3)	...	4 (5)	8 (4)	...	...	...	V
B1115	...	...	7 (4)	...	8 (4)	10 (3)	10 (3)	...	V
B1117	...	...	0 (6)	...	0 (6)	3 (6)	0 (6)	...	LV
B1118	...	...	0 (6)	...	0 (6)	2 (4)	1 (3)	...	LV
B1119	...	...	0 (6)	...	2 (5)	1 (4)	1 (3)	...	LV
B1120	...	...	7 (4)	...	8 (3)	10 (2)	10 (3)	...	V
B1121	...	...	5 (4)	...	10 (5)	10 (2)	10 (3)	...	V
B1122	...	...	10 (3)	...	10 (2)	10 (3)	10 (3)	...	V
B1123	...	...	7 (4)	...	8 (4)	10 (2)	10 (2)	...	V
B1124	...	...	6 (5)	...	9 (3)	10 (4)	10 (3)	...	V
B1125	...	...	5 (6)	...	9 (4)	10 (4)	10 (3)	...	V
B1126	...	...	0 (6)	...	2 (6)	5 (6)	7 (6)	...	V
B1127	...	...	0 (6)	...	2 (5)	4 (3)	9 (4)	...	V
B1128	...	...	0 (6)	...	0 (6)	0 (6)	0 (6)	...	AV
B1130	...	...	0 (6)	...	0 (6)	0 (6)	0 (6)	...	AV
B1131	...	...	2 (4)	...	1 (2)	10 (3)	9 (3)	...	V

V = virulent; LV = low virulence; AV = avirulent.

Separation could be made, however, by taking into account general patterns of results rather than relying on the reactions of a few specific tests. Thus, *B. anthracis* strains commonly gave results different from those of other members of the *B. cereus* group in at least five of the tests (including the four listed above). The tests useful in separating *B. anthracis* from *B. cereus* were ADH, Simmons' citrate, glycerol, salicin, cellobiose, and motility; from *B. mycoides*—which characteristically produces rhizoid growth on solid media—Simmons' citrate, glycerol, salicin and cellobiose; from *B. thuringiensis* ADH, Simmons' citrate, glycerol, salicin, cellobiose, motility and crystalline inclusions in sporangia; and from *B. cereus* strains of serotypes 1, 3, 5, 8 and 12 (headed *B. cereus* 'E' in table III) Simmons' citrate, glycerol, starch, glycogen and motility.

Table III shows that the avirulent and slightly virulent strains of *B. anthracis* had test patterns similar to those of the virulent strains and so were easily distinguished from other members of the *B. cereus* group. There was only one suggestion of a relation

TABLE III
Results of API, morphological, and supplementary tests on *Bacillus* strains

Test	Percentage of positive results given by the stated number of strains of							
	<i>B. cereus</i> (86)	<i>B. mycoides</i> (25)	<i>B. thuringiensis</i> (53)	<i>B. cereus</i> E* (30)	<i>B. anthracis</i> (all) (37)	<i>B. anthracis</i> V† (23)	<i>B. anthracis</i> LV (6)	<i>B. anthracis</i> AV (5)
API 20E:								
ONPG	0	32	0	0	0	0	0	0
Arginine dihydrolase	59	36	87	17	0	0	0	0
Citrate (Simmons')	81	60	94	100	0	0	0	0
Urease	24	20	13	0	0	0	0	0
Voges-Proskauer	94	92	98	100	100	100	100	100
Gelatin liquefaction	100	100	100	100	70	78	66	40
Nitrate reduction	84	76	92	87	100	100	100	100
API 50CH; acid from:								
Glycerol	90	96	92	70	0	0	0	0
Ribose	98	76	98	93	100	100	100	100
Galactose	8	32	0	6	0	0	0	0
D-Glucose	100	100	100	100	100	100	100	100
D-Fructose	98	94	100	100	59	48	100	60
D-Mannose	9	8	43	3	0	0	0	0
Inositol	0	3	0	0	0	0	0	0
αMethyl-D-glucoside	0	12	4	0	3	4	0	0
N Acetylglucosamine	99	100	100	100	100	100	100	100
Amygdalin	2	24	2	0	0	0	0	0
Arbutin	90	84	100	60	32	17	33	80
Aesculin	100	100	98	100	97	95	100	100
Salicin	86	80	83	0	0	0	0	0
Cellobiose	81	60	72	43	0	0	0	0
Maltose	99	100	100	100	100	100	100	100
Lactose	9	8	0	0	0	0	0	0
Sucrose	44	64	55	83	100	100	100	100
Trehalose	99	92	100	100	100	100	100	100
Melezitose	1	0	4	0	0	0	0	0
Starch	96	100	94	6	97	95	100	100
Glycogen	90	100	92	10	92	87	100	100
Xylitol	0	0	0	0	3	4	0	0
βGentiobiose	9	3	4	0	0	0	0	0
D-Turanose	16	24	11	6	3	4	0	0
L-Fucose	0	0	2	3	0	0	0	0
Glucuronate	36	12	6	40	3	4	0	0
Morphological and supplementary tests:								
Mean cell width 1.3–1.4 μm	100	100	100	100	100	100	100	100
Chains of cells	96	100	100	100	100	100	100	100
Motility	96	0	100	100	0	0	0	0
Spores ellipsoidal	100	100	100	100	100	100	100	100
Spores cylindrical	14	0	8	0	0	0	0	0
Spores central or paracentral	70	84	6	30	0	0	0	0
Spores subterminal	100	100	100	100	100	100	100	100
Crystalline inclusions	0	0	94	0	0	0	0	0
Vacuoles	83	100	100	100	100	100	100	100

Tests in which all strains studied gave negative results were: API 20E—lysine decarboxylase, ornithine decarboxylase, H₂S production, tryptophan deaminase, indole; API 50CH—erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, βmethyl-xyloside, L-sorbose, rhamnose, dulcitol, mannitol, sorbitol, αmethyl-D-mannoside, melibiose, inulin, D-raffinose, D-lyxose, D-tagatose, D-fucose, D-arabitol, L-arabitol, 2 keto-gluconate, 5 keto-gluconate; morphological and supplementary tests—spores round, spores terminal, sporangia swollen, parasporal bodies present, gas from carbohydrates.

* *B. cereus* strains of serotypes 1, 3, 5, 8 and 12 including those isolated from outbreaks of emetic-type food poisoning.

† V = virulent; LV = low virulence; AV = avirulent.

between virulence and test reaction: the proportion of arbutin positive reactions given by avirulent strains was higher (80%) than that given by slightly virulent (33%) and virulent strains (17%). Many additional strains, however, gave weak reactions scored only as 1 and 2 and, therefore, excluded from the positive results (scored as 3 and above). Had they been included, the results would have been 80%, 83% and 43% respectively. These figures, taken with the small numbers of slightly virulent and avirulent strains, are not convincing.

TABLE IV
Miscellaneous tests on 34 strains of *B. anthracis*

Strain no.	Virulence	Results in the stated tests				
		Mucoid colonies on bicarbonate agar+CO ₂	M'Fadyean reaction	Texture of colonies	Growth on PLET agar	Phage sensitivity
B0463	V	+†	(+)‡	T§	+	+¶
B0464	LV	—	—	T	+	(+)
B0467	V	—	+	T	+	+
B0469	AV	—	—	T/B	+	+
B0470	AV	+	—	T	+	(+)
B0471	V	(+)	+	T	+	+
B0472	V	—	—	T	+	+
B0473	V	+	—	T	+	+
B0474	LV	—	—	T	+	(+)
B0475	LV	—	—	T	(+)	+
B0476	V	+	+	T	+	(+)
B0477	V	+	(+)	T	+	+
B0478	V	(+)	(+)	T	+	(+)
B0479	V	+	+	T	+	+
B0480	V	(+)	(+)	T	+	+
B0481	V	(+)	(+)	T	+	+
B0482	V	+	+	T	+	+
B0483	AV	—	—	T	+	+
B0484	V	+	(+)	T	+	+
B1115	V	+	+	T	+	+
B1117	LV	—	—	T/B	(+)	+
B1118	LV	—	—	T	+	+
B1119	LV	—	—	T	(+)	+
B1120	V	+	+	T	+	+
B1121	V	+	(+)	T	+	+
B1122	V	+	+	T	+	+
B1123	V	+	+	T	+	+
B1124	V	+	+	T	+	+
B1125	V	+	+	T	+	(±)
B1126	V	+	—	T/B	+	+
B1127	V	+	—	T	+	+
B1128	AV	—	—	T/B	+	+
B1130	AV	—	—	B	+	+
B1131	V	++	+	T/B	+	+

See footnote to table II.

† ++ = Very mucoid, + = mucoid, (+) = < 30% of growth mucoid, — = growth not mucoid.

‡ + = Obvious capsule, (+) = capsulate organisms in minority, — = non-capsulate.

§ T = tenacious, T/B = intermediate, B = butyrous.

|| + = Growth, (+) = growth only when inoculum heavy, — = no growth.

¶ + = Clear plaque, (+) = some growth across plaque, (±) = plaque just visible, — = no plaque.

Miscellaneous tests

The formation of mucoid colonies, on a medium containing bicarbonate and serum and incubated in excess CO₂, is characteristic of virulent strains of *B. anthracis* and indicates abundant capsule production. However, as can be seen from table IV, not all of our virulent strains produced mucoid colonies. Strains B0467 and B0472, which were shown to be virulent (table II), produced no mucoid growth; and strains B0471, B0478, B0480 and B0481, also virulent, produced colonies less than 30% of which were mucoid. All but one (B0470) of the avirulent strains and all of the slightly virulent strains, produced non-mucoid colonies.

M'Fadyean's polychrome methylene blue will demonstrate *B. anthracis* capsules in fresh specimens or cultures grown in media that favour capsule production, such as blood. It can be seen from table IV that the results of the M'Fadyean reaction correlated less well with virulence than did the production of mucoid growth on bicarbonate agar in excess CO₂. Four virulent strains (B0472, B0473, B1126 and B1127) did not produce capsules as demonstrated by the M'Fadyean reaction; only one of these (B0472) did not produce mucoid colonies on bicarbonate agar. One virulent non-mucoid strain (B0467) gave a positive M'Fadyean reaction, and all avirulent and low virulence strains gave negative reactions.

Strains of *B. anthracis* cultivated on nutrient agar characteristically produce adherent colonies that show tenacity when disturbed with an inoculating loop; colonies of *B. cereus* have a butyrous texture. Most of the *B. anthracis* strains studied produced adherent colonies; two virulent strains (B1126 and B1131), one strain of low virulence (B1117), and two avirulent strains (B0469 and B1128) produced adherent butyrous colonies and one avirulent strain (B1130) produced entirely butyrous colonies.

All of the *B. anthracis* strains grew on PLET medium; three strains (B0475, B1117 and B1119), all of low virulence, grew only when a heavy inoculum was used. Knisely (1966) found that, on PLET medium, one of his strains grew poorly and only after incubation for 48 h. In our hands, this strain, included in the present study as no. B1131, grew well on PLET medium.

All of the strains of *B. anthracis* were lysed by the γ -phage of Brown and Cherry (1955); five strains (B0464, B0470, B0474, B0476 and B0478) showed some growth across the plaques, and one strain (B1125) produced a plaque that was only just visible.

Four strains of *B. cereus* produced butyrous colonies on bicarbonate agar in excess CO₂, gave negative M'Fadyean reactions, and lacked sensitivity to γ -phage. One strain produced small colonies on PLET agar and the others failed to grow.

DISCUSSION

It is generally accepted that loss of pathogenicity of *B. anthracis* occurs in culture (Smith *et al.*, 1952). This phenomenon is referred to by Chu (1952), Burdon (1956), Ivánovics and Földes (1958), Leise *et al.* (1959) and Burdon and Wende (1960). It is interesting, however, that of 16 strains used in this study that had been held in culture collections for long periods before the introduction of lyophilisation for preservation (strains B0463, B0464, B0467, B0469–B0476, B1126–B1128, B1130 and B1131), only two (B0474 and B0475) showed low virulence and four (B0469, B0470, B1128 and B1130) were avirulent. The two low-virulence strains were Gibson's "teaching" or "class" strains, chosen with safety in mind; it is known that strain B1130 was avirulent when tested by Cowles (1931), and strain B1128 was found to be virulent by the same author. Only three strains (B0469, B0470 and B1128) out of 13 appear, therefore, to have lost their virulence, but no information is available on the histories and original virulence of these organisms. It is noteworthy that the remaining 10 strains showed high virulence and that the two "class" strains of Gibson retained their low virulence rather than having become avirulent. These last two, like the other Gibson strains (table I), were maintained on slopes, apparently without subculturing, since 1938; they grew overnight when subcultured in 1978.

Gordon *et al.* (1973) were of the opinion that it might be very difficult to distinguish

between avirulent strains of *B. anthracis* and non-rhizoid variants of the usually non-motile *B. mycoides*. In the present work only four tests gave much assistance in making such a distinction. However, the fact that other tests which gave variable results for *B. mycoides* (ONPG, ADH, urease, galactose, amygdalin, and D-turanose) were nearly always negative for *B. anthracis* gave the opportunity of making the distinction on the basis of reaction pattern.

API and morphological tests clearly separated 22 of the *B. anthracis* strains considered in this paper (B0463–B0484) from other members of the *B. cereus* group in the computer taxonomies of Logan (1980) and Logan and Berkeley (1981). The virulent, slightly virulent and avirulent strains of *B. anthracis* were not separable by these methods.

It has been seen that virulent strains of *B. anthracis* can be easily separated from *B. cereus* by the miscellaneous tests and the API and morphological tests. The separation of avirulent and slightly virulent strains of *B. anthracis* from *B. cereus* by the miscellaneous tests is not so easy because they may produce non-mucoid colonies on bicarbonate agar incubated in excess CO₂, give negative M'Fadyean reactions, produce butyrous colonies on nutrient agar, and show poor growth on PLET medium—a medium upon which certain *B. cereus* strains can grow. The only tests in the present study which enabled such non-mucoid avirulent strains of *B. anthracis* to be separated from *B. cereus* were sensitivity to γ -phage—to which some strains of other *Bacillus* species, but not all strains of *B. anthracis* (Wilson and Miles, 1975), are susceptible—five API reactions, and motility—not always positive for *B. cereus* and always negative for *B. mycoides*.

It is apparent that a satisfactory identification system with a dichotomous key could not be based upon the tests described above, but clearly a comparison of the reaction pattern of an unidentified strain with the results presented in table III, with or without the aid of a computer (see Logan and Berkeley, 1984), would give an identification with a high probability of accuracy. Although all the members of the *B. cereus* group are very closely related it is clear that virulent, slightly virulent and avirulent strains of *B. anthracis* can easily be separated from other members of the group by the API tests described.

We thank all those who donated strains for study, API Laboratory Products Ltd for providing API test materials, Mrs Elaine Marchant for technical assistance, and Miss Kim McMillan for typing the manuscript.

REFERENCES

- Berkeley R C W, Logan N A, Shute L A, Capey A G 1984 Identification of *Bacillus* species. In: Bergan T (ed) *Methods in microbiology* vol 16. Academic Press, London, pp 291–328.
- Biegeleisen J Z, Cherry W B, Skaliy P, Moody M D 1962 The demonstration of *Bacillus anthracis* in environmental specimens by conventional and fluorescent antibody techniques. *American Journal of Hygiene* **75**:230–239.
- Bonventre P F, Eckert N J 1963 Toxin production as a criterion for differentiating *Bacillus cereus* and *Bacillus anthracis*. *Journal of Bacteriology* **85**:490–491.
- Brown E R, Cherry W B 1955 Specific identification of *Bacillus anthracis* by means of a variant bacteriophage. *Journal of Infectious Diseases* **96**:34–39.
- Burdon K L 1956 Useful criteria for the identification of *Bacillus anthracis* and related species. *Journal of Bacteriology* **71**:25–42.

- Burdon K L, Wende R D 1960 On the differentiation of anthrax bacilli from *Bacillus cereus*. *Journal of Infectious Diseases* **107**:224–234.
- Chadwick P 1959 Rapid identification of *Bacillus anthracis* by microscopical observation of bacteriophage lysis. *Journal of General Microbiology* **21**:631–634.
- Chadwick P 1963 Rapid screening test for *Bacillus anthracis*. *Canadian Journal of Microbiology* **9**:734–737.
- Chu H P 1952 Variation of *Bacillus anthracis* with special reference to the non-capsulated avirulent variant. *Journal of Hygiene* **50**:433–444.
- Clarke H E, Coates M E, Eva J K, Ford D J, Milner C K, O'Donoghue P N, Scott P P, Ward R J 1977 Dietary standards for laboratory animals: report of the Laboratory Animals Centre Diets Advisory Committee. *Laboratory Animals* **11**:1–28.
- Claus D, Schaab-Engels C (eds) 1977 Catalogue of strains 2nd edn. Deutsche Sammlung von Mikroorganismen, München, p 276.
- Cowan S T 1974 Cowan and Steel's Manual for the identification of medical bacteria 2nd edn. Cambridge University Press, Cambridge, p 152.
- Cowles P B 1931 A bacteriophage for *B. anthracis*. *Journal of Bacteriology* **21**:161–166.
- Gilbert R J, Parry J M 1977 Serotypes of *Bacillus cereus* from outbreaks of food poisoning and from routine foods. *Journal of Hygiene* **78**:69–74.
- Gilbert R J, Turnbull P C B, Parry J M, Kramer J M 1981 *Bacillus cereus* and other *Bacillus* species: their part in food poisoning and other clinical infections. In: Berkeley R C W, Goodfellow M (eds) The aerobic endospore-forming bacteria: classification and identification. Academic Press, London, pp 297–314.
- Gordon R E, Haynes W C, Pang C H-N 1973 The genus *Bacillus*. Agriculture handbook No. 427, United States Department of Agriculture. Washington D.C., pp 31–33.
- Green D M, Jamieson W M 1958 Anthrax and bone-meal fertiliser. *Lancet* **2**:153–154.
- Ivánovics G, Földes J 1958 Problems concerning the phylogenesis of *Bacillus anthracis*. *Acta Microbiologica Academiae Scientiarum Hungaricae* **5**:89–109.
- Knisely R F 1965 Differential media for the identification of *Bacillus anthracis*. *Journal of Bacteriology* **90**:1778–1783.
- Knisely R F 1966 Selective medium for *Bacillus anthracis*. *Journal of Bacteriology* **92**:784–786.
- Leise J M, Carter C H, Friedlander H, Freed S W 1959 Criteria for the identification of *Bacillus anthracis*. *Journal of Bacteriology* **77**:655–660.
- Logan N A 1980 Studies on the taxonomy and identification of members of the genus *Bacillus*: application of miniaturized, standardized, commercially available test systems. PhD thesis, University of Bristol.
- Logan N A, Berkeley R C W 1981 Classification and identification of members of the genus *Bacillus* using API tests. In: Berkeley R C W, Goodfellow M (eds) The aerobic endospore-forming bacteria: classification and identification. Academic Press, London, pp 105–140.
- Logan N A, Berkeley R C W 1984 Identification of *Bacillus* strains using the API System. *Journal of General Microbiology* **130**:1871–1882.
- Logan N A, Capel B J, Melling J, Berkeley R C W 1979 Distinction between emetic and other strains of *Bacillus cereus* using the API System and numerical methods. *FEMS Microbiology Letters* **5**:373–375.
- McFarland J 1907 The nephelometer: an instrument for estimating the number of bacteria in suspensions used for calculating the opsonic index and for vaccines. *Journal of the American Medical Association* **49**: 1176–1178.
- Manchee R J, Broster M G, Melling J, Henstridge R M, Stagg A J 1981 *Bacillus anthracis* on Gruinard Island. *Nature* **294**:254–255.
- Morris E J 1955 A selective medium for *Bacillus anthracis*. *Journal of General Microbiology* **13**: 456–460.
- Pearce T W, Powell E O 1951 A selective medium for *Bacillus anthracis*. *Journal of General Microbiology* **5**:387–390.
- Quesnel L B 1971 Microscopy and micrometry. In: Norris J R, Ribbons D W (eds) Methods in microbiology, vol 5A, Academic Press, London, p 93.
- Smith N R, Gordon R E, Clark F E 1952 Aerobic spore-forming bacteria. Agriculture monograph No. 16. United States Department of Agriculture, Washington D.C., p 66.

- Sterne M 1967 Distribution and economic importance of anthrax. *Federation Proceedings* **26**:1493–1495.
- Taylor A J, Gilbert R J 1975 *Bacillus cereus* food poisoning: a provisional serotyping scheme. *Journal of Medical Microbiology* **8**:543–550.
- Turnbull P C B, Jørgensen K, Kramer J M, Gilbert R J, Parry J M 1979 Severe clinical conditions associated with *Bacillus cereus* and the apparent involvement of exotoxins. *Journal of Clinical Pathology* **32**:289–293.
- Wilson G S, Miles A A (eds) 1975 Topley and Wilson's Principles of bacteriology, virology and immunity, 6 edn. Edward Arnold, London, vol 1 pp 1092–1093, vol 2 pp 2215–2216.