Journal of General Microbiology (1984), **130**, 1871–1882. *Printed in Great Britain*

Identification of *Bacillus* Strains Using the API System

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(*Received 14 December 1983; revised 23 March 1984*)

A system is described for the rapid and accurate identification of *Bacillus* isolates using a matrix of results from tests in the API 20E and API 50CHB strips and from supplementary tests. API System tests have been shown to be more reproducible than the classical tests. A taxonomy based upon API tests is in good agreement with those obtained by other methods. The results matrix can also be used in computer assisted identification.

INTRODUCTION

Although diagnostic keys and tables for *Bacillus* have been available for a long time (Gibson & Topping, 1938; Smith *et al.*, 1946; Cowan & Steel, 1965), the identification of these organisms is still considered to be complicated (Green, 1975; King & Phillips, 1978) and in many laboratories is taken no further than 'aerobic spore-forming rod', or '*Bacillus* species'. It is clear, however, from the numerous papers published and the large number of strains received for identification in this laboratory, that *Bacillus* species are of increasing importance in industry and medicine and that diagnosis to the species level is highly desirable in many instances.

The prevailing neglect of *Bacillus* identification may be attributed to two factors. Firstly, the diagnostic tests used; many of the classical tests for *Bacillus* described by Smith *et al.* (1946, 1952) and Gordon *et al.* (1973) require special media. These are very time consuming and expensive to prepare and many have short shelf lives resulting in considerable wastage if their use is infrequent. The requirement for media containing unusual ingredients increases the familiar problems of test standardization (Sneath, 1974; Sneath & Collins, 1974); inconsistent results may be obtained in consequence. Several tests take 14 d or more from pure culture to final reading and this is too long for many diagnosticians to wait. Any new scheme for *Bacillus* identification should therefore use widely available and standardized materials for performing a good number of rapid tests which give reproducible results.

The second factor leading to neglect of *Bacillus* identification is the character of the genus. *Bacillus* is an unusually wide taxon which contains most aerobic endospore-forming rods. In terms of DNA base ratios it is the equivalent of some bacterial families (Priest, 1981). Furthermore, some species are ill-defined, existing with closely related species as complexes or spectra in which the boundary of a particular species is difficult or impossible to identify. Even in well established species there is considerable variation between strains. Thus classical test schemes using few characters often do not permit identification of atypical and intermediate strains and in spite of the excellent work of Gordon and her colleagues (Smith *et al.*, 1952; Gordon, 1973; Gordon *et al.*, 1973), as well as others, it is widely agreed that there is considerable room for improvement in the taxonomy of the genus and that a study of new isolates, particularly, is important.

With these considerations in mind, work was started to produce a system for the ready and rapid identification of *Bacillus* strains. A study of a collection of 600 strains, of which 91 were unnamed, using 119 API and 20 supplementary tests, has been carried out (Logan & Berkeley, 1981). A development of this work (written later but published earlier), based on the 80 tests

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0022-1287/84/0001-1661 $02.00

showing greatest reproducibility and discriminatory value, was described by Logan (1980). Despite considerable between-strain variation in many species this study showed that the majority of taxa are quite distinct. API materials used in these trials included the 20E, 50E (both designed for identification of members of the *Enterobacteriaceae*), API ZYM and four other, non-commercially available, enzyme test strips (Logan, 1980). The 50E has now been superseded by the 50CH.

In this paper a development of these studies is described. It uses tests in the API 50CHB (*Bacillus*) system in place of those from the 50E, together with those used previously from the 20E, and a small number of morphological and supplementary characters. The enzyme strips were not used as they contribute little to identification (Logan, 1980).

METHODS

Strains and growth conditions. One thousand and seventy-five strains from culture collections, industrial, medical and veterinary specimens and from the natural environment were studied. All were checked, on inclusion in the collection, for purity, medium requirements and growth temperatures. The strains were representative of the whole genus, but the fastidious insect pathogens, *B. larvae*, *B. lentimorbus* and *B. popilliae*, and species requiring a very high or very low pH for growth (*B. alcalophilus* and *B. acidocaldarius* respectively) were omitted from this study. Except for these, type strains were included for all species on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980). The numbers of representatives of each species are shown in Table 1.

Strains were held at 4 °C, in the dark, on slopes of nutrient agar (Difco) containing 5 mg $MnSO_4.4H_2O$ l^{-1} (NA + Mn^{2+}) in order to enhance sporulation (Deutsche Sammlung von Mikroorganismen, 1977) and strains were allowed to sporulate prior to storage. For morphological studies strains were grown at 30 °C for 48 h (or 20 °C for 96 h for psychrophilic strains, 37 °C for 48 h for *B. coagulans*, and 55 °C for 24 h for thermophilic strains) on plates of NA + Mn^{2+}. For API tests strains were grown overnight (or, for psychrophiles, 48 h) on plates of nutrient agar (Difco) (NA) at the appropriate temperature. Certain strains would not grow satisfactorily on NA or NA + Mn^{2+} and special media had to be used, as follows.

Allantoin Mineral Medium (Deutsche Sammlung von Mikroorganismen, 1977) for *B. fastidiosus* contained ($g\,l^{-1}$): K_2HPO_4, 0·8; KH_2PO_4, 0·2; $MgSO_4.7H_2O$, 0·5; $CaCl_2.2H_2O$, 0·05; $FeSO_4.7H_2O$, 0·01; $MnSO_4.4H_2O$, 0·001 (increased to 0·005 for sporulation); allantoin (Sigma), 20; agar, 15; at pH 6·8.

'*Bacillus racemilacticus*' Medium (Deutsche Sammlung von Mikroorganismen, 1977) for '*B laevolacticus*' and '*B. racemilacticus*' contained ($g\,l^{-1}$): glucose, 5; peptone 5; yeast extract, 5; $CaCO_3$, 5; agar, 15; at pH 6·8. For sporulation, 5 mg $MnSO_4.4H_2O$ l^{-1} was added prior to autoclaving.

Bacillus pasteurii medium (Gibson & Gordon, 1974) was prepared by adding 10 g NH_4Cl l^{-1} to NA or NA + Mn^{2+} and adjusting the medium to pH 9 prior to autoclaving.

A strain that will grow only on such special media is unlikely to be isolated accidentally and its growth requirements will give some indication of its identity. In such cases the API and morphological tests serve principally to confirm or challenge this first identification.

Morphological and supplementary tests. Vegetative cell morphology observations were made on cultures grown overnight, or for such longer time as was necessary to obtain visible growth, on NA + Mn^{2+} or another suitable sporulation medium, at the appropriate temperature. Organisms were examined at 1000 × magnification, using phase contrast microscopy, for shape of cells, presence of chains, and for a foamy or vacuolate appearance of the cytoplasm. Widths of cells were measured using a Vickers-AEI image splitting eyepiece (Quesnel, 1971).

To determine motility, strains were grown on slopes of NA and after 6 h, or as soon as growth appeared thereafter, a loopful of the liquid at the base of the slope was examined at 1000 × magnification, by phase contrast microscopy.

It was occasionally possible to observe spores in slides prepared from cultures grown overnight on a suitable sporulation medium at the appropriate temperature, but most strains required incubation for 2 d, occasionally longer, before spore and sporangial morphology could be observed. Cultures were examined for shape of spores, their position in the sporangia, distension of the sporangia by mature spores, and for the presence of parasporal bodies and crystals. Three categories of spore shape were used, round, ellipsoidal and cylindrical; oval spores were recorded as both round and ellipsoidal, kidney shaped spores as ellipsoidal, and banana shaped spores as cylindrical. Intermediate cases were recorded as positive for both of the shapes that they lay between. There were three categories of spore position: terminal, subterminal and central or paracentral. Spores in several different positions might be observed in one culture. Sporangial swelling was only recorded as positive if the distension was substantial.

API tests. Tests in the API 20E strip and the API 50CHB (*Bacillus*) strips (API Laboratory Products, Basingstoke, Hants., UK) were used: 12 tests in the API 20E strip and 49 tests in the API 50CHB strips. The latter contain carbohydrate substrates for the detection of assimilation or acid production (according to the suspension

medium used) and have 37 substrates in common with the obsolete API 50E gallery. The tests are listed in Table 1.

Strains were grown on plates of NA, or another appropriate medium, overnight or, for psychrophiles, 48 h. Growth was harvested in 2 ml sterile normal saline and the suspension so produced was used to prepare two further suspensions: (i) for API 20E strip in 4 ml sterile normal saline to correspond to tube no. 3 of the McFarland (1907) series of standard opacities, which is approximately equivalent to 9×10^8 organisms ml^{-1}; and (ii) for API 50CHB strips in 10 ml of API 50CHEB (*Enterobacteriaceae/Bacillus*) medium, which contains ($g\,l^{-1}$) $(NH_4)_2SO_4$, 2; yeast extract, 0·5; tryptone, 1; phenol red, 0·18; with mineral base of Cohen-Bazire *et al.* (1957) 10 ml; in a phosphate buffer of pH 7·5 after sterilization, to correspond to tube no. 3 of the McFarland scale. Only the first twelve tests of the API 20E strip were inoculated, the last eight being carbohydrate tests duplicated in the API 50CHB strips.

Strips were incubated at 30 °C for 48 h and read at 24 and 48 h or, for psychrophiles, at 20 °C for 96 h, reading at 48 and 96 h or, for thermophiles, at 55 °C for 24 h reading at about 12 and 24 h. The API 50CHB strips were tilted at approximately 5°, bases of tubes uppermost, during incubation in order to trap any gas evolved.

Results were scored according to the manufacturer's instructions. A test scoring positive at either reading time was considered positive. Occasionally, tests appeared positive at the first reading but reverted to negative by the final reading; in the API 50CH gallery this was due to the production of large quantities of alkali which masked the acid production (alkaline reversion).

The tubes of the API 50CHB strips were examined for gas bubbles.

Identification by computer. Logan (1980) and Logan & Berkeley (1981) computed similarity coefficients using the general similarity coefficient (S_G) of Gower (1971). Clusters were formed by unweighted pair-group average linkage analysis (Sokal & Michener, 1958) and the results of several runs were illustrated by phenograms. These served as a basis for breaking the set of strains into groups of manageable size for principal co-ordinate analysis. The method of identification was that of Ross (1975) in which similarity coefficients are used. This is a simultaneous method and represents a development of the diagnostic table. Gower (1968) developed a technique for adding points to a principal co-ordinate analysis using the similarity coefficients between the new unit and each of the reference units; this useful supplement to identification by similarity coefficients enables the distinction between intermediate and outlying strains when working with groups.

All these facilities are available in the CLASP program (written by G. J. S. Ross, F. B. Lauckner and D. Hawkins, Rothamsted Experimental Station, Harpenden, Herts., UK) which was run on the ICL 4-75 computer at the University of Bristol.

All 475 new strains and the 91 unnamed strains from the studies of Logan (1980) and Logan & Berkeley (1981) were identified using the set of 509 named strains for reference.

RESULTS AND DISCUSSION

The results of the tests for the 1075 *Bacillus* strains are expressed as percentages of positive results for each species or group of strains in Table 1.

The information provided by certain tests is, however, of no value in identification because the results given are always negative. These tests are as follows: in the API 20E strip – lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase; and in the API 50CHB strips – erythritol, L-xylose, and L-arabitol. In addition, D-fucose and 2-ketogluconate appear to be of little value. This is a surprisingly small number of redundant tests considering that the systems were originally designed for the identification of *Enterobacteriaceae* and other Gram-negative rods. Several of the remaining tests appear to be of only moderate value because they merely support the separation achieved using tests of high discriminatory value; such support is, however, valuable in many cases because of the high within-species variation encountered in several species.

For several species, fewer than ten strains were available for study and in these instances the results in Table 1 are of reduced value for identification. In 25 species or groups, however, large numbers of strains were available and the results are considered to give an adequate indication of within-species variation (Sneath, 1978*a*; Gordon, 1981) for each test.

The results shown in Table 1 are most conveniently discussed by considering each species or group separately.

Bacillus cereus group

The positions of *B. mycoides* and *B. thuringiensis*, as species distinct from *B. cereus*, are not clear. Logan (1980) and Logan & Berkeley (1981) were unable to separate these three species. It can be seen from Table 1 that there are few differential tests available other than the familiar

Table 1. *Percentage positive results for Bacillus species using morphological, supplementary, API 20E and API 50CHB tests*

The table shows, for each species or group, the percentage of strains studied giving positive reactions in each test. Negative results (i.e. 0% positive) have been omitted for clarity.

	B. cereus	*B. mycoides*	*B. thuringiensis*	*B. cereus* (emetic)*	*B. anthracis*	*B. firmus*	*B. lentus*	*B. laterosporus*	*B. alvei*	*'B. thiaminolyticus'*	*'B. psychrosaccharolyticus'*	*B. insolitus*	*'B. carotarum'*
No. of strains ...	119	25	55	30	37	44	27	10	12	11	2	2	47
Morphological and supplementary tests													
1. Cell width (μm)‡	1·4	1·3	1·4	1·4	1·3	0·8	0·8	0·9	0·8	0·7	1·0	0·9	1·1
2. Chains of cells	96	100	100	100	100	29	70		25			50	100
3. Motility	96		100	100		100	100	100	100	100	100	100	42
4. Spores round							15					50	
5. Spores ellipsoidal	100	100	100	100	100	100	96	100	100	100	100	50	91
6. Spores cylindrical	22		20			29			17			50	26
7. Spores central/paracentral	70	84	6	30		75	52	100	58		100	100	62
8. Spores subterminal	100	100	100	100	100	100	100	90	100	100	100	100	86
9. Spores terminal						3					50		4
10. Sporangia swollen						51	30	100	92	91	100		
11. Parasporal bodies								100					
12. Crystalline inclusions			94										
13. Vacuoles	83	100	100	100	100								
14. Gas from carbohydrates													
API 20E tests													
15. ONPG		32				23	89		42	100	100	100	2
16. ADH	60	36	87	17		2							
17. LDC													
18. ODC													
19. Citrate (Simmons')	86	60	93	100		34	15	50		100		100	36
20. H_2S										82			
21. Urease	22	20	13				30		50	91		50	21
22. TDA													
23. Indole									100	100			
24. V-P	92	92	98	100	100	84	55	100	92	27		100	91
25. Gelatin	100	100	100	100	70	95	44	100	100	100	50		76
26. Nitrate	80	76	92	87	100	50	15	90		18		50	66
API 50CHB tests													
27. Glycerol	92	96	92	70		98	55	100	100	100	100	100	55
28. Erythritol													
29. D-Arabinose							4			100			4
30. L-Arabinose						4	70				100	100	57
31. Ribose	97	76	98	93	100	34	89	100	100	100	100	100	68
32. D-Xylose						4	18				100	100	15
33. L-Xylose													
34. Adonitol									100	9			
35. β-Methylxyloside							33					50	
36. Galactose	6	32		6		11	70		58	100		50	8
37. D-Glucose	100	100	100	100	100	100	100	100	100	100	100	100	98
38. D-Fructose	98	84	100	100	59	52	100	100		100	100	100	98
39. D-Mannose		8	41	3		23	96	100	42	100	100		
40. L-Sorbose													
41. Rhamnose							55					50	19
42. Dulcitol							15						
43. Inositol	4	3					22		75	91	100	50	38
44. Mannitol						84	96	100			100	100	70

Table 1 (*continued*)

	B. badius	*B. fastidiosus*	*'B. freudenreichii'*	*B. brevis*	*B. pasteurii*	*B. sphaericus*	*B. globisporus*	*'B. psychrophilus'*	*B. subtilis*	*'B. amyloliquefaciens'*	*B. licheniformis*	*B. pumilus*	*B. megaterium*	*B. circulans*	*B. macerans*	*B. polymyxa*	*B. macquariensis*	*'B. laevolacticus'*	*'B. racemilacticus'*	*B. coagulans*	*B. stearothermophilus* 1†	*B. stearothermophilus* 2†	*B. stearothermophilus* 3†	*'B. caldolyticus'* group	*B. pantothenticus*
	2	5	3	18	6	54	3	2	131	52	81	63	33	44	15	15	3	5	4	20	38	4	32	3	18
Tests																									
1.	0·9	1·3	0·9	0·9	0·7	1·0	0·9	0·9	0·8	0·8	0·8	0·7	1·5	0·8	0·7	0·9	0·6	0·7	0·7	0·8	0·8	0·7	0·9	0·8	0·6
2.	100	100	100	5	100		33	50	22	84	80	5	97	23	7	13		20	50	35	66		40		83
3.	100	100	100	100	100	100	100	100	95	100	100	100	97	100	100	100	100	40	100	100	100	100	100	100	100
4.					100	100	100	100					69												78
5.	100	100	100	100		20			100	100	100	61	100	100	100	100	100	100	100	100	87	100	100	100	78
6.									8		20	89		20	7	7				5	37		9		
7.	50	80	100	81					40	7	49	40	40	29		40					10	75	3		
8.	100	80	67	100	33	79	100	100	98	100	100	100	100	84	100	100	100			100	100	97	100	100	22
9.	50	20			100	74	67	100	15	36	12			45	33		100	100	100	55	39		53	100	100
10.			33	100	100	100	100	50	5		2			84	100	100	100	100	100	85	68	100	100	100	100
11.																									
12.																									
13.													90												
14.															100	100									
15.				22		7	100	100	90	67	100	100	100	100	100	100	100	60	50	70	26		12		78
16.		100									95							20							
17.																									
18.																									
19.	100	80	33	44		85		100	98	92	99	89	85	4											22
20.																									
21.		100	100		100	65	100	100			16			7				20	25						
22.																									
23.																									
24.				72		11		100	100	100	100	98	97	73	67	93		100	100	100	81	100	69	33	50
25.	100			33		4	67	100	100	100	100	98	100	38	40	93		20		30	95	100	100	100	94
26.			100	77	100	14		100	95	88	96		15	13	47	100		20			60	25	16		50
27.				50					97	96	100	98	100	86	100	100		80	100	100	100	100	97	100	100
28.																									
29.														23	93	7			25	10					83
30.									98	82	100	98	97	91	100	100			100	75	71	25	9		
31.				16					99	100	100	100	100	98	100	100	100	20	100	80	89	25	44	100	100
32.									89	71	99	98	91	98	100	100	100		100	70	66	75			
33.																									
34.																					3	50			
35.														93	100	100	100				3				
36.									30	44	100	100	97	100	100	100	100	100	100	95	60	50	84	100	100
37.				11				100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
38.				22		7		100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
39.									94	63	99	97	15	98	100	100	33	100	100	95	89	75	94	100	100
40.									2		7	5						100	75	5	16	25	62		
41.									2		83	5	3	45	100	33		60	50	50	8				100
42.														2								75			
43.				11					95	86	76	28	100	41	40					40	26	75			28
44.				11					96	100	90	100	100	88	100	100	100	100	100	40	79	100		100	50

Table 1 (*continued*)

	B. cereus	*B. mycoides*	*B. thuringiensis*	*B. cereus* (emetic)*	*B. anthracis*	*B. firmus*	*B. lentus*	*B. laterosporus*	*B. alvei*	*'B. thiaminolyticus'*	*'B. psychrosaccharolyticus'*	*B. insolitus*	*'B. carotarum'*
No. of strains ...	119	25	55	30	37	44	27	10	12	11	2	2	47
API 50CHB tests (continued)													
45. Sorbitol						7	52				50	50	21
46. α-Methyl-D-mannoside						2	15			91		50	
47. α-Methyl-D-glucoside	2	12	4		3	2	52		67	100	100	100	
48. *N*-Acetylglucosamine	99	100	100	100	100	88	92	100	100	100	100	100	47
49. Amygdalin	8	24	2				70	70	50	100	100	100	4
50. Arbutin	91	84	100	60	32	13	81	100	67	100	100	100	13
51. Aesculin	100	100	98	100	97	73	100	100	100	100	100	100	34
52. Salicin	87	80	83			13	78	100	42	100	100	100	19
53. Cellobiose	84	60	72	43		7	81	90	50	91	100	100	81
54. Maltose	98	100	100	100	100	100	100	100	100	100	100	100	62
55. Lactose	8	8				2	67		25	91	100	50	2
56. Melibiose						2	78		50	100	100	50	4
57. Sucrose	47	64	55	83	100	91	100		75	100	100	100	68
58. Trehalose	98	92	100	100	100	75	89	100	58	100	100	50	89
59. Inulin						7	11					50	40
60. Melezitose	1		4			2	48		17	100		50	2
61. D-Raffinose			1			7	89		50	100	100	50	42
62. Starch	96	100	94	6	97	91	100	50	100	100	100	50	36
63. Glycogen	92	100	92	10	92	54	70		67	91	100	50	36
64. Xylitol					3		11						
65. β-Gentiobiose	18	3	4			7	63	90	83	100	100	100	15
66. D-Turanose	15	24	11	6	3	13	63		67	100	100	100	2
67. D-Lyxose							7					50	
68. D-Tagatose						2	15					50	
69. D-Fucose													
70. L-Fucose			2	3			4		8	100			17
71. D-Arabitol						4	15			9		50	
72. L-Arabitol													
73. Gluconate	29	12	6	40	3		26		8	100	50		
74. 2-Ketogluconate													
75. 5-Ketogluconate							4		17	100			

rhizoid growth of *B. mycoides* and its lack of motility, and the pathogenicity for insects of *B. thuringiensis* and its production of crystalline inclusions. The results suggest that *B. mycoides* and *B. thuringiensis* should be considered as varieties of *B. cereus*.

Bacillus cereus strains of serotypes 1, 3, 5 and 8 (which include strains isolated in connection with outbreaks of emetic type food poisoning: Gilbert & Parry, 1977; Gilbert *et al.*, 1981; Taylor & Gilbert, 1975) gave characteristic results which enable their separation from other members of the *B. cereus* group (Logan *et al.*, 1979); they are listed as *B. cereus* (emetic) in Table 1.

The 37 strains of *B. anthracis* comprised five avirulent, six low virulence, 23 virulent and three strains of unknown virulence; it is not possible to distinguish between these using API tests.

Table 1 (*continued*)

Tests	*B. badius*	*B. fastidiosus*	*B. freudenreichii*	*B. brevis*	*B. pasteurii*	*B. sphaericus*	*B. globisporus*	*B. psychrophilus*	*B. subtilis*	*B. amyloliquefaciens*	*B. licheniformis*	*B. pumilus*	*B. megaterium*	*B. circulans*	*B. macerans*	*B. polymyxa*	*B. macquariensis*	*B. laevolacticus*	*B. racemilacticus*	*B. coagulans*	*B. stearothermophilus* 1†	*B. stearothermophilus* 2†	*B. stearothermophilus* 3†	*B. caldolyticus* group	*B. pantothenticus*
	2	5	3	18	6	54	3	2	131	52	81	63	33	44	15	15	3	5	4	20	38	4	32	3	18
45.									88	88	95	17	54	25	73			100	100	35	21	100	9		78
46.												92	6	18	100	40	67	80	100	20			44		
47.									99	98	100	55	60	95	100	100	100	100	100	85	60	100	87	100	100
48.						54	33	100	22	19	89	100	97	79	20	20	100	100	100	95	47		6		100
49.									99	100	100	97	100	98	100	100	100	100	100	70	18	100	16	67	100
50.									100	100	100	100	100	98	100	100	100	100	100	65	55	100	3	67	100
51.				16		2	33		100	100	100	100	100	100	100	100	100		75	95	76	100	22	100	100
52.									100	100	100	100	100	100	100	100	100	100	100	65	71	100	34	100	100
53.									100	100	100	100	100	98	100	100	100	100	100	70	63	100	37	100	94
54.				5		4			100	98	100	46	100	100	100	100	100	100	100	100	100	100	93	100	100
55.									49	84	89	76	100	100	100	100	100	100	100	85	13	25	50		50
56.									80	56	45	23	100	100	100	100	100	100	100	90	58		87	100	5
57.									100	100	100	100	100	98	100	100	67	100	100	85	97	100	100	100	89
58.						4			100	82	100	100	100	98	100	100	100	100	100	100	97	100	90	100	100
59.									83	11	68	1	85	70	100	87		100	75	5					
60.											1		69	52	100	40		100	75	5	81	25	100	100	
61.									90	92	79	89	100	98	100	100	100	100	100	95	60	25	97	100	
62.				5		4			98	98	99		100	95	100	100	100	100	100	95	89		100	100	100
63.				5					98	94	96		100	93	100	100	100	60	50	35	47		90	100	17
64.				5							1		30	4							3	100			
65.									96	88	89	100	97	98	100	100	100		25	70	37	100	56	100	55
66.									97	79	100	54	100	100	100	100	100	100	75	95	95	100	100	100	100
67.																7		60	75						
68.								100			96	65	3	2				100	50	45	79	75	87	100	100
69.													3						25						
70.													3	43	87							100			78
71.													60	7	80	7			25	55		100			
72.																7					3				
73.									4		2		15	61	93	80	67		50	75					72
74.														2				20		30					
75.									2				6	79	93	53		60	50	55	89	100	100		

* *Bacillus cereus* strains isolated in connection with outbreaks of emetic-type food poisoning and strains of serotypes 1, 3, 5 and 8 commonly associated with such outbreaks.

† Groups 1 to 3 of Walker & Wolf (1971).

‡ Mean cell width for strains studied.

Bacillus firmus and B. lentus

A spectrum-like arrangement of strains of *B. firmus*, *B. lentus* and pigmented isolates (SM strains) from salt marsh and sea water (Turner & Jervis, 1968) was observed by Gordon *et al.* (1977) and discussed by Logan & Berkeley (1981). The species results in Table 1 incorporate results of tests on the SM strains; these were allocated to the species to which they showed greatest similarity. The great within-species and between-species variation of *B. firmus* and *B. lentus* and the extent to which they have common characters is evident in the results shown, but

differentiation is possible because *B. lentus* strains produce acid from a wider range of carbohydrates.

Bacillus laterosporus

This is a species with highly characteristic biochemical and morphological features and the strains examined showed little variation.

Bacillus alvei and 'B. thiaminolyticus'

Although the results shown in Table 1 suggest some similarity between the two groups and only a few strains of each have been studied, it is clear that they may be separated easily using API tests. As Gibson & Gordon (1974) point out, more strains of these organisms need to be studied.

'Bacillus carotarum'

Considerable between-strain variation is seen in this group and its homogeneity is in doubt. Logan (1980) and Logan & Berkeley (1981) found '*B. carotarum*' to exist as a series or spectrum of strains and Gibson (1935) observed considerable morphological variation in strains allocated to this species. Several of the strains studied were received as *B. megaterium* or *B. cereus–B. megaterium* intermediates and came from the N. R. Smith collection. Smith *et al.* (1946, 1952) regarded '*B. carotarum*' as a synonym of *B. megaterium* but this was rejected by Gibson & Gordon (1974), whose decision was supported by Bonde (1973, 1975), Hunger & Claus (1978, 1981), Logan (1980) and Logan & Berkeley (1981). None of the strains studied in the present work showed appreciable similarity to *B. cereus* or *B. megaterium*.

Mesophilic species showing little or no acid production from carbohydrates

The species *B. badius*, *B. fastidiosus*, '*B. freudenreichii*', *B. brevis*, *B. pasteurii* and *B. sphaericus* all produce little or no acid from carbohydrates and the few positive results gained from tests in the API 50CHB strips are of little importance in differentiation. The patterns of results given in the API 20E strip, the characteristic vegetative cell and sporangial morphologies, and the special media requirements of certain species (*B. fastidiosus* and *B. pasteurii*) make separation simple.

Psychrophilic species

Of the five psychrophilic groups considered ('*B. psychrosaccharolyticus*', *B. insolitus*, *B. globisporus*, '*B. psychrophilus*' and *B. macquariensis*), none is represented by more than three strains; the results shown are, therefore, of limited value in identification. Within-species variation is low, except in the case of *B. insolitus*, and the patterns of results are individual enough to support the status of each species.

The Bacillus subtilis group and B. megaterium

The *B. subtilis* spectrum or group as defined by Gibson (1944) and Gordon *et al.* (1973) comprises *B. subtilis*, *B. pumilus* and *B. licheniformis*. To this may be added '*B. amyloliquefaciens*' which was regarded by Gordon *et al.* (1973) as a synonym of *B. subtilis*, a position formalized in the Approved Lists (Skerman *et al.*, 1980). Logan (1980) and Logan & Berkeley (1981) found that *B. megaterium* clustered with the *B. subtilis* group and so it too is considered here.

O'Donnell *et al.* (1980) studied eight strains of each of the four species comprising the *B. subtilis* group and found that they could separate *B. subtilis*, *B. pumilus*, *B. licheniformis* and '*B. amyloliquefaciens*' using API tests, DNA reassociation studies and pyrolysis gas liquid chromatography but that '*B. amyloliquefaciens*' could not be separated from *B. subtilis* when the classical tests of Gordon *et al.* (1973) were used. Study of a larger number of strains by Logan (1980) and Logan & Berkeley (1981) revealed the presence of intermediate strains which obscured the distinction so that the species cannot now be clearly separated using the API tests. Only two tests in Table 1, acid production from inulin, and chains of cells, are of value in separating the two species.

The separation of the other species presents no difficulty, despite appreciable within-species variation.

Bacillus circulans, B. macerans and B. polymyxa

Gibson & Topping (1938) found *B. circulans* to be a species complex and later workers have endorsed this description (Logan & Berkeley, 1981). The results summarized in Table 1 for the 44 strains of this species studied indicate great between-strain variation. The pattern of reactions is, however, individual enough to enable *B. circulans* to be separated from the two closely related species *B. macerans* and *B. polymyxa*. Logan (1980) and Logan & Berkeley (1981) found *B. macerans* to be so closely related to *B. circulans* and *B. polymyxa* that it lay between and almost joined them in principal co-ordinate plots.

Bacillus coagulans, 'B. laevolacticus' and 'B. racemilacticus'

The species *B. coagulans* forms a very diffuse group showing great variation between strains so that a species pattern of reactions is difficult to define. Other authors have also found the species to be heterogeneous (Logan & Berkeley, 1981). '*Bacillus laevolacticus*' and '*B. racemilacticus*' were originally separated mainly on the basis of the isomer, or isomers, of lactic acid produced by glucose fermentation (Gibson & Gordon, 1974); their similarity is clear from the results, albeit of few strains, shown in Table 1, but separation is possible. The close relationship of these two species to *B. coagulans* is also clear but, again, differentiation is possible.

Bacillus stearothermophilus and the 'B. caldolyticus' group

The results of Logan (1980) and Logan & Berkeley (1981) supported the division of *B. stearothermophilus* into the three groups proposed by Walker & Wolf (1971); the results given for *B. stearothermophilus* in Table 1 are divided into the three groups accordingly. Group 1 is represented by only four strains but appears fairly homogeneous. Although groups 1 and 3 are heterogeneous (Walker & Wolf subdivided these groups) and there are few tests available for differentiation each has a characteristic pattern of results. It is possible that further study will show that the members of these groups represent a discontinuous series or spectrum.

The species '*B. caldolyticus*', '*B. caldotenax*' and '*B. caldovelox*' (Heinen & Heinen, 1972), each represented by a single strain, show great similarity and a close relationship to *B. stearothermophilus* group 3.

Bacillus pantothenticus

In the analyses of Logan (1980) and Logan & Berkeley (1981) this species showed phenotypic relatedness to *B. coagulans* and *B. stearothermophilus*; it is, however, a homogeneous species with characteristic morphological and biochemical properties that allow it to be differentiated from these two species with ease.

Other species

The foregoing covers 26 of the 31 *Bacillus* species included in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980). The remaining five species are *B. larvae*, *B. lentimorbus*, *B. popilliae*, *B. alcalophilus* and *B. acidocaldarius*; the first three of these are fastidious and require special growth media and conditions that are more easily supplied in the classical test procedures. *Bacillus alcalophilus* and *B. acidocaldarius* require very high and very low pH, respectively, for growth and are not, therefore, compatible with the narrow pH ranges of the media in API strips. These organisms may, however, be identified satisfactorily by the nature of their requirements for growth.

A taxon-radius model type of identification system, for use with a computer, is easily constructed from a table of per cent positive results (Sneath, 1978*b*). The method assumes, however, that the taxa are roughly hyperspherical and that intermediate forms are relatively uncommon; the model breaks down if there is considerable overlap of hyperspheres (Sneath & Sokal, 1973) but this would indicate the need for, and may be remedied by, reclassification. Clearly, the spectra and species complexes occurring in *Bacillus* (*B. firmus*, *B. lentus* and *B.*

circulans for example) cannot be regarded as hyperspherical and appreciable overlap of taxa occurs. Furthermore, a minimum of ten strains should be used for each taxon in such systems (Sneath, 1978*b*) and several of the taxa in the present work do not satisfy this requirement.

Discriminant analysis is an extension of the taxon-radius model method and gives reliable identification with groups that show partial overlap in their properties; it achieves this by weighted transformation of character axes so that average within-taxon scatter is standardized, and by altering angles between character axes to make clusters as hyperspherical as possible. This advantage over the taxon-radius model, however, is counteracted by the method requiring the taxa to be few in number, the clusters to be of similar size, shape and orientation, and the unknown to belong to one of the taxa included (Sneath & Sokal, 1973; Sneath, 1978*b*). The first and last of these problems are substantially overcome by the use of the generalized distance function D^2 (Mahalanobis, 1936; Sneath & Sokal, 1973) which is used in canonical variates analysis. Such methods, however, have limited application; large numbers of individuals are required in each taxon but the gain in discrimination over simpler methods may be slight (Sneath & Sokal, 1973).

A large number of taxa are considered in the present work; this, taken with the inadequate representation of some of these taxa, argues against the use of discriminant analysis. On the other hand, the difficulties of separating several of the groups and the high degree of phenetic overlapping that occurs with such polythetic taxa make sequential methods, such as dichotomous keys and polyclaves, inappropriate (Sneath & Sokal, 1973).

It may be concluded, therefore, that simultaneous methods such as the use of diagnostic tables, identification by similarity coefficients, and, for the clearly defined and adequately represented taxa, taxon-radius models are most suited to the task of identifying members of the genus *Bacillus*.

Other identification schemes for *Bacillus* such as those of Gibson & Topping (1938), Smith *et al.* (1946, 1952), Wolf & Barker (1968), Gordon (1973), and Gordon *et al.* (1973), based upon dichotomous keys, and those of Cowan & Steel (1965, 1974) based upon tables, do not allow the identification of so many species as the system described here and usually do not permit the identification of atypical and intermediate strains. The keys of Gordon *et al.*, (1973) and Bonde (1978, and personal communication) for example, use 27 and 22 characters and recognize only 21 and 10 taxa respectively. The computer-assisted identification system described by Willemse-Collinet *et al.* (1980), and based upon studies of 30 strains from culture collections, used 24 tests, taking up to 7 d for the identification of only 18 species, and was unable to achieve a satisfactory diagnosis in several instances, although misidentification did not occur. Our results enable the recognition of 38 taxa and the availability of results for a large number of tests (Table 1) allows the identification of atypical and intermediate strains. Establishment of the validities of some of the taxa recognized awaits the results from studies on more strains and of further information from other sources. The system does, however, make possible the diagnosis of 26 of the 31 *Bacillus* species included in the Approved Lists of Bacterial Names (Skerman *et al.*, 1980).

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