Numerical classification and identification of *Bacillus sphaericus* including some strains pathogenic for mosquito larvae

BRIAN ALEXANDER and FERGUS G. PRIEST*

Department of Biological Sciences, Heriot Watt University, Riccarton, Edinburgh EH14 4AS, UK

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Ninety-one strains of *Bacillus sphaericus*, including representatives of all the established DNA homology groups, related round-spored and oval-spored species, and six strains pathogenic for mosquito larvae, were examined for 155 characters. Numerical analyses (Jaccard coefficient/average linkage clustering) based on the 88 variable features revealed 14 clusters at the 79% similarity level that contained more than one strain and 17 single member clusters. All insect pathogenic strains were recovered in a single cluster and the classification was in accord with an established classification based on DNA sequence homology. Two frequency matrices for probabilistic identification were constructed and tested. A comprehensive matrix comprising 14 mesophilic, round-spored taxa and 27 tests gave good results for identification of hypothetical median organisms, cluster overlap and identifications of representative strains (based on data generated in the classification study). Reference strains for the 14 taxa and eight additional insect pathogenic strains were examined for the 27 tests and were correctly identified with high scores using this matrix. A second matrix comprising seven taxa and 13 tests also performed well in the theoretical evaluation and correctly identified the reference strains and insect pathogenic strains.

Introduction

Aerobic, rod-shaped bacteria that differentiate into spherical spores have been classified into several species within the genus *Bacillus*. Ribosomal RNA analyses indicate that these bacteria may represent a separate evolutionary line within the genus (Stackebrandt *et al.*, 1987), and it is notable that most strains are atypical in cell-wall peptidoglycan structure, containing lysine or ornithine rather than *meso*-2,6-diaminopimelate (*meso*-DAP; Schleifer & Kandler, 1972) which is typical of *B. subtilis* and oval-sporing relatives (Bartlett & White, 1985). These bacteria are also characterized by negative reactions to most of the traditional phenotypic tests used for the classification and identification of bacilli. This largely arises from their obligately aerobic physiology (an exception is the alkaliphile *B. pasteurii*) and inability to use sugars as sources of carbon and energy (Russell *et al.*, 1989). As a result, mesophilic, round-spored bacilli that grow at neutral pH are invariably labelled *B. sphaericus* and the species appears phenotypically homogeneous. This was apparent in a recent numerical classification in which 13 strains were assigned to two closely linked taxa (Priest *et al.*, 1988).

Abbreviations: HMO, hypothetical median organism; *meso*-DAP; *meso*-2,6-diaminopimelate.

The heterogeneity of *B. sphaericus* has been revealed by DNA homology studies in which 50 strains were allocated to six homology groups and 12 strains remained ungrouped (Krych *et al.*, 1980). These homology groups have recently been substantiated by multilocus enzyme electrophoresis (Singer, 1988) but diagnostic phenotypic characters have yet to be determined. This is particularly important because members of DNA homology group IIA are pathogenic for some mosquito larvae, in particular certain *Aedes*, *Culex* and *Mansonia* species, and have considerable potential as biological control agents (World Health Organization, 1985; Singer, 1987). At present, these strains can only be distinguished by DNA homology, enzyme electrophoresis or toxicity testing, all of which require a certain level of laboratory sophistication. A small battery of diagnostic phenotypic tests should allow simple identification of potential pathogenic strains.

In this study we have examined 91 strains representing all the *B. sphaericus* DNA homology groups, related reference strains and 32 environmental isolates for 155 phenotypic features. The data were analysed using standard numerical taxonomic procedures and frequency matrices are presented for the probabilistic identification of *B. sphaericus* taxa including the insect pathogenic types.

Methods

Strains and isolation conditions. The strains and their sources are given in Table 1. Bacteria were isolated from soil and mud samples using several selective procedures designed for the isolation of *B. sphaericus* strains. All cultures were incubated at 30 °C unless otherwise stated. Thermophiles were incubated at 50 °C and psychrophiles at 10 °C. (a) For acetate enrichments, soil suspensions (1 g in 5 ml water) were heated at 60 °C for 30 min and incubated in 50 ml A3 medium

(Massie *et al.*, 1985) for 24 h with shaking. Samples were plated onto solidified A3 medium and incubated for 48 h. Single colonies from samples were purified and retained.

(b) For adenine enrichments, the same procedure as (a) was followed but the (NH₄)₂SO₄ in A3 medium was replaced by adenine (1 g l⁻¹). Enrichments were plated and isolates purified on adenine agar.

(c) Creatinine enrichments were based on A3 medium with acetate replaced by citrate (5 g l⁻¹) and 2.5 ml 20% (w/v) creatinine l⁻¹ in place of (NH₄)₂SO₄ as nitrogen source. Enrichment broths were plated and isolates purified on creatinine agar or nutrient agar as indicated.

Table 1. Designation and sources of strains assigned to clusters defined in Fig. 1 (at 79% S_i, UPGMA)

Binomials in inverted commas are not on the Approved Lists of Bacterial Names (Skerman *et al.*, 1980) and have not been validly published since 1 January 1980. Type strains are marked with an asterisk (*). Strains prefixed 'S' have been described previously (Priest *et al.*, 1988). Other strains were isolated using acetate (Ac), adenine (Ad), creatinine (Cr), creatinine-nutrient agar (Cr-NA), chloramphenicol/lincomycin (Cl) and BATS medium (see Methods) from woodland soil in E. Lothian, Scotland, UK and Corfu, Greece and mud from River Tyne (E. Lothian) and Smeaton Loch (E. Lothian).

Cluster 1 (<i>Bacillus sphaericus sensu stricto</i> , DNA homology group I)		Cluster 8	
BS1-5	<i>B. sphaericus</i> , DSM 28,* NCIB 9370,* ATCC 14577,*† NCTC 9602,† ATCC 10208†	BS39, 40, 42, 43	<i>B. sphaericus</i> , 19/2,‡ 19/3,‡ III/2,‡ III/3‡
BS28, 29	<i>B. sphaericus</i> , S304, S305	BS96	Cluster 9
BS84, 116	Woodland soil, E. Lothian, Ac, Cr	BS99	Mud from River Tyne, BATS
BS87, 144	Mud from River Tyne, Ac		Mud from Smeaton Loch, BATS
BS89, 91, 145, 146	Mud from Smeaton Loch, Ac		Cluster 10
	Cluster 2	BS81	Mud from Smeaton Loch, Ad
BS27	<i>B. sphaericus</i> , S303	BS82, 83	Woodland soil, Corfu, both Ad
BS30, 31	<i>B. sphaericus</i> , S306, S307	BS85	Woodland soil, E. Lothian, Ac
BS100	Mud from Smeaton Loch, BATS		Cluster 11
Subcluster 3a (mosquito pathogens, DNA homology group IIA)		BS33, 44, 45	<i>B. sphaericus</i> , N. See 1b,‡ VI/2a,‡ VI/2b‡
BS6-11	<i>B. sphaericus</i> , S. Singer 1593,† DSM 1867, SSII-I,† Kellen K,† 2297,† 2362†	BS37, 38	Cluster 12
	Subcluster 3b (<i>Bacillus fusiformis</i> , DNA homology group IIB)		<i>B. sphaericus</i> , 6/1,‡ 19/1‡
BS12-14	<i>B. sphaericus</i> , ATCC 7055,*† DSM 493, ATCC 12300†	BS48, 49	Cluster 13 (thermophilic strains)
	Subcluster 3c		<i>B. sphaericus</i> , DSM 461, DSM 462
BS76	Woodland soil, E. Lothian, Ad	BS70, 71	Cluster 14 (<i>Bacillus psychrophilus</i>)
BS93, 117, 125, 127	Woodland soil, Corfu, Ac, Ac, Cr, Cl		<i>B. psychrophilus</i> , DSM 3,* S434
	Cluster 4 (DNA homology group V)	BS25	Single-member clusters
BS23, 24	<i>B. sphaericus</i> , NRS 1198,† NRS 1199†	BS32	<i>B. sphaericus</i> , NRS 1186‡
	Cluster 5 (DNA homology group III)	BS34	(DNA homology ungrouped)
BS15-18	<i>B. sphaericus</i> , NRS 592,† ATCC 4525,† P1,† DSM 2368	BS41	<i>B. sphaericus</i> , N. See 1a‡
BS26	<i>B. sphaericus</i> , S302	BS46	<i>B. sphaericus</i> , lineola‡
BS106, 107	Woodland soil, E. Lothian, Cr, Cr-NA	BS53	<i>B. sphaericus</i> , II/1‡
BS109, 110	Mud from River Tyne, Cr-NA, Cr	BS54	<i>B. sphaericus</i> , VI/3‡
BS112, 113, 115	Mud from Smeaton Loch, Cr-NA, Cr, Cr	BS55	' <i>B. loehnisii</i> ', DSM 488
	Cluster 6 (DNA homology group IV)	BS58	' <i>B. aminovorans</i> ', DSM 1314
BS19, 20, 22	<i>B. sphaericus</i> , NRS 400,† NRS 1693,† ATCC 13805†	BS59	<i>B. pasteurii</i> , DSM 33*
BS75, 79	Woodland soil, E. Lothian, both Ad	BS64	' <i>B. macroides</i> ', DSM 54
BS80	Mud from River Tyne, Ad	BS65	<i>B. badius</i> , DSM 23*
	Cluster 7	BS66	' <i>B. repens</i> ', S295
BS35, 36	<i>B. sphaericus</i> , 1/2,‡ 1/3‡	BS67	' <i>B. sphaericus</i> var. <i>rotans</i> ', S299
		BS68	<i>Bacillus</i> sp., S353
		BS69	<i>Bacillus</i> sp., S365
		BS74	<i>B. globisporus</i> , DSM 4*
			<i>B. marinus</i> , DSM 1297*
			Woodland soil, E. Lothian, Ad

† A. A. Yousten, Microbiology Section, Biology Department, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA.

‡ Strain from DSM, Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG.

Table 2. Percentage distribution of positive diagnostic characters to mesophilic clusters defined at 79% level (S_1)

Cluster number ... DNA homology group* ... Number of strains ...	Taxon													
	1	2	3a	3b	3c	4	5	6	7	8	9	10	11	12
	I	—	IIA	IIB	—	V	III	IV	—	—	—	—	—	—
	15	4	6	3	5	2	12	6	2	4	2	4	3	2
<i>Degradation of:</i>														
1. Adenine†	1	1	67	1	1	1	1	1	1	1	1	1	1	1
2. Casein†	67	25	99	99	80	50	8	1	1	1	99	25	1	1
3. Gelatin†	93	50	99	99	99	99	1	99	1	1	99	25	1	1
4. Urea†	1	1	99	99	99	99	58	17	1	99	1	1	1	1
<i>Resistance to ($\mu\text{g ml}^{-1}$)</i>														
5. Cephalixin (5)	13	50	83	67	99	50	83	50	99	99	1	1	99	1
6. Chloramphenicol (8)	7	25	99	99	99	1	83	1	1	25	50	1	1	1
7. Erythromycin (1)†	13	50	1	99	99	1	99	99	99	50	1	1	99	99
8. Fusidic acid (5)	27	99	99	99	99	99	99	99	99	99	99	1	99	50
9. Streptomycin (150)†	99	99	99	99	80	99	1	99	1	1	99	99	33	1
10. Tetracycline (2)	67	99	99	99	99	50	75	83	50	50	99	1	1	50
11. Trimethoprim (30)†	99	99	99	99	99	1	92	86	99	99	99	99	99	50
12. Tobramycin (5)	7	99	1	67	40	99	99	1	99	50	99	1	1	1
<i>Utilization of (0.2% w/v):</i>														
13. Glycerate	13	75	99	99	1	1	42	50	99	1	50	1	67	50
14. α -Oxoglutarate	33	75	33	1	80	99	75	33	99	75	1	25	33	1
15. Tartrate	13	75	1	1	60	1	50	17	1	1	1	1	1	1
<i>Utilization of (0.4% w/v):</i>														
16. D- α -Alanine	93	99	67	1	99	1	99	67	99	1	1	1	1	1
17. L- α -Alanine	87	50	83	1	99	1	58	67	1	1	1	1	1	99
18. Citraconate†	1	1	67	99	1	1	1	1	99	1	1	1	1	1
19. Cytosine	99	99	99	99	99	99	99	99	99	99	99	33	1	1
20. Ethanolamine	99	99	33	1	99	1	83	99	99	1	1	1	99	99
21. L-Isoleucine	99	25	1	1	60	99	67	50	1	75	1	1	1	1
22. L-Leucine†	99	99	99	99	99	1	1	33	99	99	1	1	1	1
23. L-Serine††	99	99	1	33	80	99	99	99	99	99	50	1	99	99
24. L-Threonine††	99	99	50	1	99	99	99	99	99	99	50	99	67	1
<i>Growth in (% w/v):</i>														
25. NaCl (7)	1	1	99	99	99	50	50	67	50	75	99	1	33	99
26. Thallous acetate (0.01)†	73	75	99	99	99	1	1	1	99	50	1	25	1	1
27. Crystal violet (0.0001)	47	25	99	33	60	1	99	83	99	99	99	50	99	99
<i>Growth at:</i>														
28. pH 5.0	67	50	99	99	99	99	99	99	99	99	25	1	1	1
<i>Miscellaneous tests:</i>														
29. Chymotrypsin	99	75	99	99	99	99	92	33	1	50	1	1	99	99

* DNA homology groups of Krych *et al.* (1980)

† The 13 characters tested used in the smaller matrix for clusters 1, 3a, 3b, 3c, 4, 5 and 6.

‡ Characters used only in the 13 test matrix.

(d) BATS medium was inoculated directly with pasteurized soil samples as described by Yousten *et al.* (1985).

(e) Streptomycin/lincomycin agar (Kalfon *et al.*, 1986) was inoculated directly with pasteurized soil. Lincomycin was replaced by chloramphenicol ($6 \mu\text{g ml}^{-1}$) in streptomycin/chloramphenicol agar and chloramphenicol/lincomycin agar contained chloramphenicol ($6 \mu\text{g ml}^{-1}$) instead of streptomycin. Both were inoculated directly with pasteurized soil.

All strains were purified by replating and stored as cell/spore suspensions in 20% (v/v) glycerol at -20°C .

Phenotypic tests. Bacteria were grown on nutrient agar for 48 h and

visibly turbid suspensions were prepared in saline (0.85%). Media in Petri dishes, or Replidishes (Sterilin), as appropriate (see Priest *et al.*, 1988), were inoculated using a multipoint inoculator (Denley) and incubated for 5 d unless stated otherwise. Test procedures have been described in detail (Priest *et al.*, 1988); additions and amendments where appropriate are given below.

Morphological tests. The following were of little or no diagnostic value: colonies flat/raised, smooth, rhizoidal, entire, opaque, motile or pigmented; cell length; ends pointed; Gram reaction; spore shape; spore position or swelling of sporangium.

Degradation tests. The following characters had little or no diagnostic

potential. Hydrolysis of: carboxymethylcellulose (Alexander & Priest, 1989); chondroitin (0.4% in nutrient agar), detected by flooding plates after incubation for 4 d with 1% (w/v) cetyltrimethylammonium bromide; creatinine (detected as an alkaline reaction after incubation for 2 d in Christensen's urease medium with the urea replaced by 1% w/v, filter-sterilized creatinine); DNA; elastin; hypoxanthine; testosterone; Tween 20; Tween 80; degradation of uric acid and xanthine. Tests with diagnostic value are given in Table 2.

Antibiotic resistance tests. All antibiotics were supplied by Sigma and included in nutrient agar at the concentrations indicated in Table 2. Good growth after incubation for 4 d was scored as positive. The following antibiotics ($\mu\text{g ml}^{-1}$) were of little or no diagnostic value: cephaloridine (25), clindamycin (5), colistin sulphate (25), cycloserine (128), gentamycin (5), gramicidin (64), kanamycin (10), lincomycin (30), nalidixic acid (64), nitrofurantoin (25), novobiocin (5), penicillin (4), polymyxin (16), rifampicin (0.125), spectinomycin (5), sulphonamide (30) and vancomycin (5).

Utilization of organic acids. The medium of Gordon *et al.* (1973) was used. Growth accompanied by a change in indicator colour after incubation for 4 d was recorded as a positive reaction. The list of organic acids tested comprises the characters given in Table 2 and the following, which were of little or no diagnostic value: acetate, butyrate, hydroxybutyrate, citrate, formate, fumarate, gluconate, hydroxymethyl glutarate, lactate, malate, pyruvate and succinate.

Utilization of sole sources of carbon. The organic acid medium was supplemented with various carbon sources (Table 2 and see below) at 0.4% (w/v). Growth after incubation for 4 d indicated a positive response. Tests for utilization of the following had poor or no diagnostic value: acetamide, adipic acid, β -alanine, *m*-aminobenzoate, DL-aminobutyrate, amylamine, anthranilate, L-arginine, L-asparagine, betaine, L-aspartate, choline citrate, L-citrulline, ergosterol, glycerol, glycine, glucosamine, D-glucose, L-glutamate, L-glutamine, L-histidine, L-hydroxybenzoate, levulinate, mesaconate, nicotinamide, L-norvaline, L-ornithine, L-proline, D-ribose, L-tyrosine and L-valine.

Tolerance tests. Growth in the presence of various compounds (Table 2 and see below) was examined using nutrient agar supplemented appropriately; growth after incubation for 4 d was considered a positive response. The following had no diagnostic value [growth in the presence of (% w/v)]: phenol (0.1), phenylethanol (0.1, 0.3), potassium tellurite (0.01, 0.001), sodium chloride (5, 10), sodium azide (0.01, 0.02) and thallos acetate (0.001). Tests for growth at 20, 40 and 50 °C on nutrient agar, and at pH 5.5, 6.0 and 6.5 showed little diagnostic potential.

Enzyme substrates. Ability to hydrolyse a range of nitrophenyl sugar derivatives was examined according to the method described by Cowan (1974) for *o*-nitrophenyl β -D-galactoside hydrolysis using 96-well microtitre trays. Cells were incubated with substrate for 18 h before addition of 0.5 M-sodium carbonate. The following substrates were uniformly negative: *p*-nitrophenyl (*pN*)-acetate, *pN*-anthranilate, *pN*- α -L-fucopyranoside, *pN*- α -D-galactopyranoside, *oN*- β -D-galactopyranoside, *pN*- α -D-glucopyranoside, *pN*- β -D-glucopyranoside, *pN*- β -D-glucuronide, *pN*-laurate, *pN*- α -D-mannopyranoside, *pN*- β -D-mannopyranoside, *pN*-myristate, *pN*- α -D-xylopyranoside and *oN*- β -D-xylopyranoside. Chymotrypsin- and trypsin-like activities were detected by incubating multipoint inoculated nutrient agar plates for 48 h and overlaying with semi-solid agar containing *N*-glutaryl-L-phenylalanine-*p*-nitroanilide and *N*-benzoyl-DL-*p*-nitroanilide (both 1 mg ml⁻¹). A yellow colour after incubation for 6 h was scored positive.

Miscellaneous tests. Cells were analysed for the presence of lysine in cell walls using the method described by Kandler & Weiss (1986). The following tests had little or no diagnostic potential: reduction of nitrate,

phenylalanine deamination, phosphatase activity, arginine dihydrolase activity, dihydroxyacetone reaction and indole production.

Data analysis. Twelve strains were duplicated and test results were analysed according to Sneath & Johnson (1972) to provide an assessment of test error. Results for tests that were either positive or negative for all strains were excluded from the computation. The final matrix therefore comprised data for 91 strains and 88 tests. Data were scored in binary code and analysed using the Clustan IC package Release 2 (Wishart, 1978) on a Vax cluster computer. Two proximity coefficients (Jaccard, S_J , and simple matching, S_{SM}) were calculated and similarity matrices were clustered using the unweighted pair group method with arithmetic averages (UPGMA) algorithm (see Sneath & Sokal, 1973 for details). Cophenetic correlation was determined for the two dendrograms using Clustan.

The frequency matrix (Table 2) was analysed using CHARSEP (Sneath, 1979b) and DIACHAR (Sneath, 1980a) which calculate character separation indices and most diagnostic tests for clusters, respectively. From this information, two frequency matrices were prepared for probabilistic identification. The larger matrix was tested for cluster overlap using OVERMAT (Sneath, 1980c) and hypothetical median organisms (HMOs) were calculated and identified using MOSTTYP (Sneath, 1980b). Test results were redetermined for cluster representatives and some additional strains, which were identified using MATIDEN (Sneath, 1979a). Use of these programs has been described previously (Priest & Alexander, 1988).

Results

Numerical classification

Results for the 12 duplicated strains indicated an overall probability of test error of 1%. The two computations gave slightly different classifications as indicated below. The relationships between clusters differed slightly in that cluster 4 of the S_J analysis was recovered between clusters 8 and 9 in the S_{SM} dendrogram (Fig. 1).

The 91 strains were recovered in 14 clusters containing more than one strain and 17 single member clusters at the 79% S_J level which corresponded to 14 clusters and 12 single member clusters at 87% S_{SM} . The two dendrograms showed 0.952 and 0.935 cophenetic correlation respectively and consequently the S_J /UPGMA dendrogram was considered to reflect the most accurate classification. Table 2 does not include data for the two thermophilic and two psychrotrophic strains (clusters 13 and 14 respectively in Fig. 1) since the study concentrates on the classification and identification of mesophilic strains likely to be recovered under normal isolation conditions. Nevertheless, these bacteria and the oval-spored reference strains are included in Fig. 1 to demonstrate the relationships between them.

Both dendrograms closely resembled the classification based on DNA homology determined by Krych *et al.* (1980). The following description will concentrate on the S_J /UPGMA analysis. Cluster 1 contained the type strain of

Fig. 1. Simplified dendrogram showing the relationship between clusters recovered in the S_1 /UPGMA analysis.

B. sphaericus and strains belonging to DNA homology group I. This cluster represents *B. sphaericus sensu stricto*. These strains hydrolysed casein, gelatin and Tween 80 and were resistant to high levels of streptomycin and

trimethoprim. They did not hydrolyse urea but grew on a range of organic acids and amino acids, notably acetate and arginine which are used in selective media for these bacteria. Indeed, of the eight environmental isolates in

this cluster, seven were isolated using acetate as a selective carbon source.

Cluster 2 contained three strains from our laboratory collection that had clustered with *B. sphaericus* in a previous study (Priest *et al.*, 1988), and an environmental isolate. In the S_{SM} computation it also contained strains BS35 and BS36 which were supplied by the DSM and had been isolated from soil using casein enrichments. The few tests which separated this cluster from cluster 1 included tartrate and glycerate utilization and tobramycin resistance.

Cluster 3 was divided into three subclusters in accord with the DNA homology groups reported by Krych *et al.* (1980). All the insect pathogenic strains (DNA homology group IIA) included in the study were recovered in subcluster 3a in both computations. These joined subcluster 3b at the 85.5% S_j level corresponding to 90% S_{SM} . Subcluster 3b contained strains from DNA homology group IIB (Krych *et al.*, 1980) that represent *B. fusiformis*. The major distinction between strains of subclusters 3a and 3b was the ability of most strains in subcluster 3a to clear adenine from nutrient agar. Indeed, no other strains in the study could do this. Subcluster 3a strains were resistant to streptomycin and chloramphenicol which are used in selective media for these bacteria. They could be distinguished from *B. sphaericus sensu stricto* by the ability to hydrolyse urea and, for most strains, to utilize citrate. Strains of subcluster 3c were isolated using a variety of media, with all but one originating from woodland soil from Corfu, Greece. The subcluster was recovered intact in both computations and recent results (unpublished) show that it comprises a separate DNA homology group.

DNA homology group V strains were recovered as cluster 4 and the 12 strains in cluster 5 represent DNA homology group III. Of these 12 strains, seven were environmental isolates from creatinine enrichments and the remainder were reference strains. Cluster 6 contained three reference strains from DNA homology group IV and three environmental isolates from adenine enrichments. Clusters 4, 5 and 6 could be distinguished using gelatin hydrolysis, resistance to erythromycin, streptomycin, trimethoprim and tobramycin, growth on ethanolamine, D-alanine and glucosamine and resistance to crystal violet (Table 2).

There were some inconsistencies amongst the two cluster 7 strains which were included in cluster 2 in the S_{SM} dendrogram. Both were received from the DSM and originated from casein enrichments. They differed from cluster 2 strains in being resistant to gramicidin, sensitive to streptomycin and failing to grow on tartrate, but showing growth on citraconate and adipic acid. Their status as a separate cluster therefore seems to be valid.

The remaining clusters comprised environmental isolates or strains isolated from casein enrichments and supplied by the DSM. Cluster 9 contained an additional strain (BS72) in the S_{SM} computation and cluster 10 strains were mainly isolated using adenine enrichments. Two thermotolerant, round-spored strains were recovered in cluster 13. These bacteria grew at 55 °C but not at 37 °C. The *B. psychrophilus* strains were assigned to cluster 14. These bacteria grew only below 25 °C.

Single-member clusters largely comprised reference strains of round- and oval-spored species. Strain BS25 was ungrouped in both this study and the DNA homology study of Krych *et al.* (1980). Round-spored strains that failed to cluster included '*B. loehnisii*', '*B. aminovorans*', '*B. pasteurii*', '*B. repens*', '*B. sphaericus* var. *rotans*', *B. globisporus* and *B. marinus*. Oval-spored strains considered to be physiologically related to *B. sphaericus* but which were unclustered included '*B. macroides*' and *B. badius*. All round-spored strains included in the study with the exception of '*B. aminovorans*' contained lysine in their cell walls and *meso*-DAP was not detected. Conversely, the cell walls from the oval-spored strains and '*B. aminovorans*' contained *meso*-DAP.

Identification

The data in Table 2 were used to prepare two matrices for probabilistic identification. We used 14 clusters/subclusters but excluded the thermophiles and psychrophiles since such strains would not be isolated using incubation conditions for *B. sphaericus* of 30 or 37 °C. The 88 differential characters were evaluated using CHARSEP which provides five different separation indices for assessing the diagnostic value of characters including the VSP index (Sneath, 1979b) which, for useful characters, will be greater than 25%. Twenty-seven characters that possessed VSP > 48.25% (except adenine degradation, VSP 1% but essential for identification of cluster 3a) were chosen for the matrix (see Table 2). The 14 taxa in the matrix showed little overlap as assessed by the OVERMAT program (cluster 1/cluster 2 = 3.2%; remainder < 1%) and standard deviations for clusters were below 0.245 indicating that they were compact. HMOs were calculated and identified using MOSTTYP. All but one identified with Willcox probabilities of 1.0, the exception being a score of 0.999 for cluster 2. Taxonomic distance (*D*) was below 0.257 and standard error of taxonomic distance (*SED*) below -1.07 for the HMOs of all clusters. Sample strains (representing strains from the centres and peripheries of clusters) were identified using the data generated in the numerical classification. All identified with Willcox probabilities > 0.999 (*D* < 0.446 and *SED* < 6.0). When the test data were re-determined for these strains the values dropped to > 0.986 for

Table 3. Identification coefficients for type or reference strains using the two identification matrices

Cluster number*	Reference strain	Willcox probability†	Taxonomic distance (D)†	SED†
1. (DNA I)	DSM 28‡	1.000 (1.000)	0.311 (0.125)	0.177 (-2.178)
2.	S307	0.999	0.328	-0.322
3a. (DNA IIA)	1593	0.999 (0.999)	0.180 (0.189)	-1.659 (-1.075)
3b. (DNA IIB)	ATCC 7055‡	1.000 (0.999)	0.110 (0.186)	-2.855 (0.867)
3c.	BS126	1.000 (0.999)	0.222 (0.174)	-1.297 (0.189)
4. (DNA V)	NRS 1198	1.000 (0.999)	0.192 (0.196)	-0.599 (-0.374)
5. (DNA III)	NRS 592	1.000 (0.999)	0.375 (0.183)	1.658 (-1.040)
6. (DNA IV)	NRS 400	0.999 (0.999)	0.343 (0.312)	0.700 (1.345)
7.	1/3	0.999	0.239	-0.531
8.	III/2	0.998	0.348	0.581
9.	BS99	0.999	0.233	2.227
10.	BS82	1.000	0.147	-2.538
11.	VI/2a	0.999	0.213	-0.172
12.	6/1	0.999	0.371	0.381

* DNA homology groups of Krych *et al.* (1980) are shown in parentheses.

† Figures in parentheses are identification coefficients obtained with the small (13 character) matrix.

‡ Type strain.

Table 4. Identification coefficients for some mosquito pathogenic strains of *B. sphaericus*

Strain*	Identification	Willcox probability†	Taxonomic distance (D)†	SED†
WHO 1883	Taxon 3a (DNA IIA)	0.999 (0.988)	0.266 (0.147)	0.815 (-1.944)
WHO 2013-6	Taxon 3a (DNA IIA)	0.999 (0.999)	0.424 (0.216)	5.590 (-0.526)
WHO 2115	Taxon 3a (DNA IIA)	0.984 (0.971)	0.266 (0.347)	0.815 (2.181)
WHO 2173	Taxon 3a (DNA IIA)	0.999 (0.999)	0.379 (0.346)	4.221 (2.155)
WHO 2314-2	Taxon 3a (DNA IIA)	0.996 (0.988)	0.380 (0.392)	4.250 (3.107)
WHO 2315	Taxon 3a (DNA IIA)	0.999 (0.999)	0.364 (0.236)	3.768 (-0.114)
WHO 2377	Taxon 3a (DNA IIA)	0.999 (0.999)	0.395 (0.392)	4.713 (3.107)
WHO 2500	Taxon 3a (DNA IIA)	0.999 (0.999)	0.379 (0.147)	4.221 (-1.944)

* All strains were supplied by A. A. Yousten, Microbiology Section, Biology Department, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, USA.

† Figures in parentheses are identification coefficients obtained using the small (13 character) matrix.

Willcox probabilities and <0.459 and <7.0 for *D* and *SED*, respectively. These poor values for *D* and *SED* were for clusters 7, 9 and 12 and clusters 1 to 6 consistently obtained good scores (<0.436 and <4.13, respectively). Type or reference strains were re-tested for the 27 characters. All identified well to their clusters (Table 3). Some strains supplied as probable insect pathogens were also examined for the 27 tests and all identified to subcluster 3a (DNA homology group IIA) with acceptable scores (Table 4).

When identifying field isolates suspected to be insect pathogens, the full battery of 27 tests may not be necessary. We tested a second matrix based on the clusters that represent the established DNA homology groups only and taxon 3c. The matrix therefore comprised seven taxa (clusters 1, 3a, 3b, 3c, 4, 5 and 6) and 13 tests (Table 2). This matrix performed remark-

ably well. Overlap was below 1% for all taxa (using OVERMAT) and HMOs identified with high scores (Willcox probabilities 0.998-1.0). Identification coefficients for cluster representatives were acceptable before and after re-determination of the characters, and all reference strains were identified (Table 3). Of particular note, suspected insect pathogenic strains identified to cluster 3a (DNA homology group IIA) with high scores (Table 4).

Discussion

Studies of DNA homology and enzyme electrophoresis have consistently revealed diversity amongst round-spored bacilli, despite apparent phenotypic homogeneity (Krych *et al.*, 1980; Singer, 1988). Some of the taxa

within *B. sphaericus* can be distinguished by using test methods based on carbon-source utilization rather than acid production from carbohydrates (de Barjac *et al.*, 1980; Baumann *et al.*, 1984) but the use of such methods alone does not enable complete phenotypic demarcation of the DNA homology groups. The combination of carbon-source utilization tests and other physiological and biochemical characters used in this study has provided a phenotypic classification that is completely compatible with that based on DNA homology and has enabled the separation of several clusters (e.g. 1, 2 and 5) which contain strains that in a previous study were combined as *B. sphaericus* (Priest *et al.*, 1988). Thus in this study, each of the homology groups was recovered in its entirety and each probably represents a species.

The insect pathogenic strains were all allocated to subcluster 3a. This could be considered a distinct species since it is phenotypically and genetically distinct from *B. sphaericus sensu stricto* (cluster 1), but the separation from *B. fusiformis* (subcluster 3b) is poor. There is still a lack of good diagnostic characters for subcluster 3a; clearance of adenine from nutrient agar, citrate utilization and sensitivity to erythromycin are the three most useful features. Nevertheless, insect pathogenic strains were consistently assigned to subcluster 3a using both identification matrices. It is interesting to compare the relationships within cluster 3 with the situation pertaining to the other important insect pathogenic spore-former, *B. thuringiensis*. *B. thuringiensis* strains are virtually indistinguishable from *B. cereus* using phenotypic features but numerical classification reveals separate subclusters at about the 90% similarity level (Priest *et al.*, 1988). Moreover, there is considerable DNA homology between strains of *B. thuringiensis* and *B. cereus* (Somerville & Jones, 1972) and, like 3a and 3b strains, thermal denaturation studies suggest that this represents poorly matched hybrids. Crystal-protein genes are generally plasmid-borne in *B. thuringiensis* (Aronson *et al.*, 1986) but, although many of the pathogenic strains of '*B. sphaericus*' contain one or several large plasmids (Singer, 1988), the locations of the crystal-protein genes have yet to be determined (Baumann *et al.*, 1987; Hindley & Berry, 1987). Finally, *B. thuringiensis* strains can be allocated to serotypes (de Barjac, 1981). Similarly, strains within subcluster 3a have been grouped by phage typing (Yousten, 1984) and serology (de Barjac *et al.*, 1980) and the groups correlate well with insect toxicity. Since the name *B. thuringiensis* has been retained for what many people believe to be 'insect pathogenic strains of *B. cereus*', and this has value for insect pathologists, so it could be argued that the insect pathogenic strains related to *B. fusiformis* be given species status.

We isolated strains from soils in Eastern Scotland that

clustered with DNA homology group I strains (cluster 1 strains; *B. sphaericus sensu stricto*), group III strains (cluster 5) and group IV strains (cluster 6) but not homology groups IIA, IIB or V (subclusters 3a, 3b and 4 respectively). This may be due to inappropriate selective isolation, since cluster 1 environmental isolates, with one exception, were recovered using acetate as principal carbon source; cluster 5 strains were all isolated from creatinine enrichments, and cluster 6 strains from adenine plates. Alternatively, inability to recover cluster 3a, 3b and 4 strains from local samples might reflect scarcity of these strains in northern latitudes; all insect pathogens to date have been derived from either diseased insect larvae or soil/mud samples from countries having Mediterranean-type or tropical climates (Lysenko *et al.*, 1985; Brownbridge & Margalit, 1987).

Although the enrichment of cluster 5 strains with creatinine seems to contradict the phenotypic test in which all strains were recorded negative for creatinine hydrolysis, the latter was tested as an alkaline reaction. Presumably, these strains do not hydrolyse creatinine using the iminohydrolase pathway that produces *N*-methylhydantoin and ammonia from the substrate (Szulmajster, 1958; Esders & Lynn, 1985) and use the reactions involving *N*-methylhydantoin and sarcosine as exemplified by *Clostridium sordellii* (Möller *et al.*, 1986), or some other pathway which does not result in an appreciable increase in pH.

Other enrichment methods did not prove particularly successful in this study. Strains isolated on BATS medium, which is intended to be selective for insect pathogens (Yousten *et al.*, 1984), were recovered in clusters 2 and 9. Streptomycin/lincomycin (Kalfon *et al.*, 1986) and streptomycin/chloramphenicol agars failed to recover any round-spored strains and the isolates BS125 and BS127 from chloramphenicol/lincomycin agar were recovered in cluster 3c. Again, it is possible that the failure of lincomycin/streptomycin and BATS media was due to the scarcity of insect pathogenic strains in our local samples. Nevertheless, there is certainly a need for better selective media. Since only the mosquito pathogens cleared adenine from nutrient agar plates (Table 2), we are currently evaluating various medium formulations based on purines and their derivatives.

Cluster patterns of the reference strains were in accord with other studies (Claus & Berkeley, 1986; Priest *et al.*, 1988). The only round-spored strain that contained meso-DAP in its cell wall was '*B. aminovorans*' and this strain showed very little phenotypic similarity to the other round-spored strains. Ribosomal RNA cataloguing also shows that this bacterium has diverged significantly from *B. sphaericus* (Stackebrandt *et al.*, 1987). Recovery of *B. marinus*, '*B. aminovorans*' and *B. globisporus* in separate clusters substantiates other studies (Rüger,

1983; Nakamura, 1984) and confirms the existence of several species with round spores. Similarly, the absence of phenetic similarity with the oval-spored strains indicates that the separate lineages of oval- and round-spored strains is reflected in their phenotypes.

The classification described here formed the basis of a reliable identification system. The large matrix should prove useful for the general identification of mesophilic bacilli that form round spores. The theoretical evaluation of this matrix proved it to be robust, and the practical testing using reference strains indicated that it will be of general use. The smaller matrix, on the other hand, should be of great value to those involved in identifying insect pathogenic strains. The scores obtained with this matrix were acceptable, being well within the limits for identification scores set in other studies (e.g. Williams *et al.*, 1985; Priest & Alexander, 1988). It is anticipated that this matrix will be particularly useful when identifying restricted selections of strains recovered, for example, from selective isolation plates or diseased insects.

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