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Phylogeny of *Bacillus sphaericus*-like organisms

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The mesophilic round-spored bacteria embrace four species, namely *Bacillus sphaericus*, *Bacillus fusiformis*, *Bacillus silvestris* and *Bacillus pasteurii*. Although not displayed by all strains, mosquito pathogenicity is a noteworthy characteristic of *B. sphaericus sensu lato*. Phylogenetic analysis based on 16S rDNA sequences from 58 strains identified as *B. sphaericus* was used to examine the genetic heterogeneity of the taxon. Results from sequence analysis were compared with whole-cell fatty acid profiles and other phenotypic determinations. The *B. sphaericus*-like strains segregated into seven distinct clusters in a phylogenetic tree generated from 16S sequences. One cluster represented *B. sphaericus* and another *B. fusiformis*. A third cluster containing all of the pathogenic strains was closely related to, or was possibly part of, the *B. fusiformis* group. The remaining four groups were distinct and represented unnamed taxa that were more closely related to *B. sphaericus* and *B. fusiformis* than to the psychrophilic round-spored species, *Bacillus globisporus* and *Bacillus psychrophilus*. Groups based on phenotypic analysis corresponded to the 16S rDNA phylogenetic clusters. Data showed that *B. sphaericus* was genetically and phenotypically a highly heterogeneous taxon including at least seven genetically distinct taxa. The pathogenic strains were members of a distinct group and not of the species *B. sphaericus sensu stricto*. This heterogeneity partially accounts for the apparent variability of mosquito pathogenicity among *B. sphaericus* strains.

Keywords: phylogeny, *Bacillus sphaericus*, taxonomy, 16S rRNA, insect pathogen

INTRODUCTION

The earliest round-spored species, *Bacillus pasteurii*, was an alkalophile described by Chester in 1898. Subsequently, Neide (1904) described the mesophilic round-spored species, *Bacillus sphaericus*. Because of poor reactivity to classical biochemical tests, all mesophilic, round-spored organisms were assigned to the species *B. sphaericus*. In 1988, Priest *et al.* described another species, namely *Bacillus fusiformis*; its urea-hydrolysing capability differentiated it from *B. sphaericus*. Because of the lack of differentiating tests, the taxonomic study of round-spored bacteria languished. However, with the discovery of mosquitocidal activity among some of these organisms (Kellen *et al.*, 1964) and the development of diagnostic molecular biological techniques, interest in these organisms has been rekindled. Application of the new techniques has resulted in the discovery of the newest round-spore species, *Bacillus silvestris* (Rheims *et al.*, 1999). Numerical taxonomic techniques (Priest *et al.*, 1988; Alexander & Priest, 1990) suggested the heterogeneity of *B. sphaericus*; the observation of variability in insecticidal activity among strains reinforced this view. Using DNA hybridization techniques, Krych *et al.* (1980) separated the species into several subgroups only one of which contained the insecticidal strains. Later studies based on random amplified polymorphic DNA (RAPD) probing (Woodburn *et al.*, 1995) and ribosomal gene restriction fragment length polymorphism (RFLP) analyses (Aquino de Muro *et al.*, 1992) confirmed the separate groupings.

Analytical comparison of the 16S rRNA gene sequences has provided a sensitive technique for unravelling the phylogenetic relationships among microorganisms. The present study applies sequencing combined with biochemical and physiological charac-

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Abbreviation: RAPD, random amplified polymorphic DNA.

The GenBank accession numbers for the 16S rDNA sequences generated in this study are listed in Table 1.

terization methods to study the taxonomy and phylogeny of *B. sphaericus*-like organisms.

METHODS

Bacterial strains. The bacterial strains used in this study were isolated from a variety of environments throughout the world (Table 1). The strains are maintained by the Agricultural Research Service Culture Collection (NRRL), National Center for Agricultural Utilization Research. The NRRL B- prefixes denote strains obtained directly from a source or isolated at the Center; BD- and NRS- prefixes denote strains obtained from the collections of B. Delaporte and N. R. Smith, respectively. Working stock cultures were

Table 1. *Bacillus sphaericus*-like organisms used in this study

NRRL no.	Received as:	Source*	History†	GenBank no.
Group 1				
B-14957	SSII-1	1	Pathogenic; (BS8, Alexander & Priest); Krych group = IIA; RFLP group = [RIIA] RIIA; RAPD group = DNA IIA	AF169492
B-23269	DSM 1867	2	A. Yousten ← S. Singer, 1593; pathogenic, from dead mosquito; (BS6, Alexander & Priest); Krych group = IIA; RFLP group = [RIIA] RIIA; RAPD group = DNA IIA	AF169496
B-23279	IEBC S01.001	3	Kellen K; from *Culex incidens*; (BS9, Alexander & Priest); pathogenic	AF169497
B-23280	IEBC S01.002	3	Kellen Q; from *Culex incidens*	AF169498
B-23281	IEBC S02.020	3	17N; isolated in New Caledonia	AF169499
B-23282	IEBC S05.001	3	S. Singer, 1593; *Culex fatigans*; pathogenic; Krych group = IIA; RFLP group = [RIIA] RIIA; RAPD group = DNA IIA	AF169500
B-23283	IEBC S05.007	3	2217-2; isolated in the Philippines; pathogenic	AF169501
B-23284	IEBC S05.016	3	B41; isolated in India; pathogenic	AF169502
B-23285	IEBC S05.030	3	Bake 1; from cesspool, Ivory Coast; pathogenic	AF169503
B-23286	IEBC S05.055	3	2601; from grasshopper, Hungary; pathogenic	AF169504
B-23287	IEBC S05.110	3	O3; from *Culex pipiens*; pathogenic	AF169505
B-23288	IEBC S06.030	3	B55; from insect, India	AF169506
BD-111	45.E.1	4	From mud; Nahazari, India	AF169512
Group 2				
B-14865	5d-4	5	From soil	AF169490
B-14905	19	6	From marine environment	AF169491
NRS-156	NRS-156	7	F. D. Mauer, Army Medical Center; Krych group = IIB	AF169530
NRS-350^T	NRS-350	7	AMNH, *B. fusiformis* 732; (ATCC 7055^T; BS12, Alexander & Priest); Krych group = IIB; RFLP group = RIIB RIIB; RAPD group = DNA IIB	AF169537
NRS-400	NRS-400	7	From Colorado soil; (BS19, Alexander & Priest); Krych group = IIB; RFLP group = RIV (RIII); RAPD group = DNA IIB	AF169538
NRS-718	NRS-718	7	From soil; Krych group = IIB; RAPD group = DNA IIB	AF169540
NRS-866	NRS-866	7	J. R. Porter, *B. fusiformis* F15 ← NCTC 2608 ← W. W. Ford 24D; (ATCC 10300)	AF169546
NRS-732	NRS-732	7	ATCC 245, '*Bacillus lactimorbus*'	AF169542
NRS-1223	NRS-1223	7	D. H. Rose 6A; from orange; Krych group = III	AF169529
Group 3				
B-183		8	'*B. rotans*'; Krych group = unknown	AF169493
B-23268	DSM 28^T	2	*B. sphaericus* type strain; ATCC 14577^T; (BS1-5, Alexander & Priest); Krych group = I; RFLP group = RI RI; RAPD group = DNA I	AF169495
NRS-967	NRS-967	7	J. R. Porter, S14a ← N. H. Clausen; Krych group = I; RAPD group = DNA I	AF169547
BD-83	9.A.12	4	From soil; Cote d'Ivoire	AF169518
BD-94	19.B.5	4	From soil; Grenada, Antilles	AF169523

Table 1 (*cont.*)

NRRL no.	Received as:	Source*	History†	GenBank no.
BD-95	20.B.11	4	From soil; Joshua Tree National Monument, California	AF169524
BD-107	45.B.6	4	From soil; Nahazari, India	AF169510
Group 4				
B-4297	8.10114	9	J. R. Norris; Krych group = III	AF169507
NRS-111	NRS-111	7	W. Bohrer, M-7; isolated from corn; (ATCC 12123)	AF169526
NRS-593	NRS-593	7	NIH, clinical pathogen; Krych group = III	AF169539
NRS-719	NRS-719	7	R. P. Tittsler, '*B. pseudotetani*'; Krych group = III	AF169541
NRS-800	NRS-800	7	C. Lamana, A20; Krych group = III	AF169544
NRS-810	NRS-810	7	J. R. Porter, *B. alvei* A102G. Bredemann ← C. Neide; Krych group = III	AF169545
NRS-1692	NRS-1692	7	J. R. Norris, '*B. pycnoticus*' ← T. Gibson 6; Krych group = III	AF169532
BD-115	48.C.4	4	From soil; Grand Nord, Canada	AF169514
BD-117	50.D.5	4	From soil; Grand Nord, Canada	AF169515
BD-119	54.E.3	4	From soil; Grand Nord, Canada	AF169516
BD-121	58.D.5	4	From soil; Font Romeu, Pyrenees	AF169517
Group 5				
B-1876	NRS-717	7	From angleworm intestine; Krych group = unknown	AF169494
NRS-250	NRS-250	7	From soil; Krych group = unknown	AF169536
NRS-752	NRS-752	7	A4; from soil exposed to chloropicrin	AF169543
NRS-1186	NRS-1186	7	H. W. Reuszer Army 875; (BS25, Alexander & Priest); Krych group = unknown	AF169527
NRS-1198	NRS-1198	7	H. W. Reuszer Army 883; Krych group = V; RFLP = RV RV; RAPD = DNA V	AF169528
BD-89	18.B.10	4	From soil; Trinidad, Antilles	AF169521
BD-93	19.B.4	4	From soil; Grenada, Antilles	AF169522
BD-109	45.B.12	4	From mud; Nahazari, India	AF169511
BD-113	45.E.7	4	From mud; Nahazari, India	AF169513
Group 6				
NRS-1691	NRS-1691	7	J. R. Norris, '*B. pycnoticus*' ← T. Gibson 4; Krych group = unknown	AF169531
NRS-1693	NRS-1693	7	J. R. Norris, '*B. pycnoticus*' ←T. Gibson 10; Krych group = IV; (BS20, Alexander & Priest); RFLP = (RIII) RIII; RAPD = DNA IV	AF169533
NRS-1694	NRS-1694	7	J. R. Norris, '*B. pycnoticus*' ← T. Gibson 13; Krych group = unknown	AF169534
NRS-1695	NRS-1695	7	J. R. Norris, '*B. pycnoticus*' ← T. Gibson 14	AF169535
BD-85	11.F.6	4	From soil; Cote d'Ivoire	AF169519
Group 7				
BD-87	16.M.1	4	From soil; Norway	AF169520
BD-99	22.B.1	4	From soil; Grand Nord, Canada	AF169525
BD-101	22.B.7	4	From soil; Grand Nord, Canada	AF169508
BD-103	22.C.6	4	From soil; Grand Nord, Canada	AF169509

*Sources are as follows: 1, A. A. Yousten, Virginia Polytechnic Institute and University, Blacksburg, VA, USA; 2, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ), Braunschweig, Germany; 3, Bacteries Entomopathogenes Institut Pasteur, Paris, France; 4, B. Delaporte, Institut Pasteur, Paris, France; 5, J. Huang, National Center for Agricultural Utilization Research, Peoria, IL, USA; 6, R. Slepecky, Syracuse University, Utica, NY, USA; 7, N. R. Smith, US Department of Agriculture, Beltsville, MD, USA; 8, E. McCoy, University of Wisconsin, Madison, WI, USA; 9, J. R. Norris, University of Leeds, Leeds, UK.

†Abbreviations are as follows: AMNH, American Museum of Natural History; NIH, National Institutes of Health; ATCC, American Type Culture Collection; designations in parentheses are equivalent strain numbers. Krych (DNA similarity) groups are from Krych *et al*. (1980), RFLP groups are from Aquino de Muro *et al*. (1992) and RAPD groups are from Woodburn *et al*. (1995). Names in quotation marks are not on the Approved Lists of Bacterial Names (Skerman *et al*., 1980).

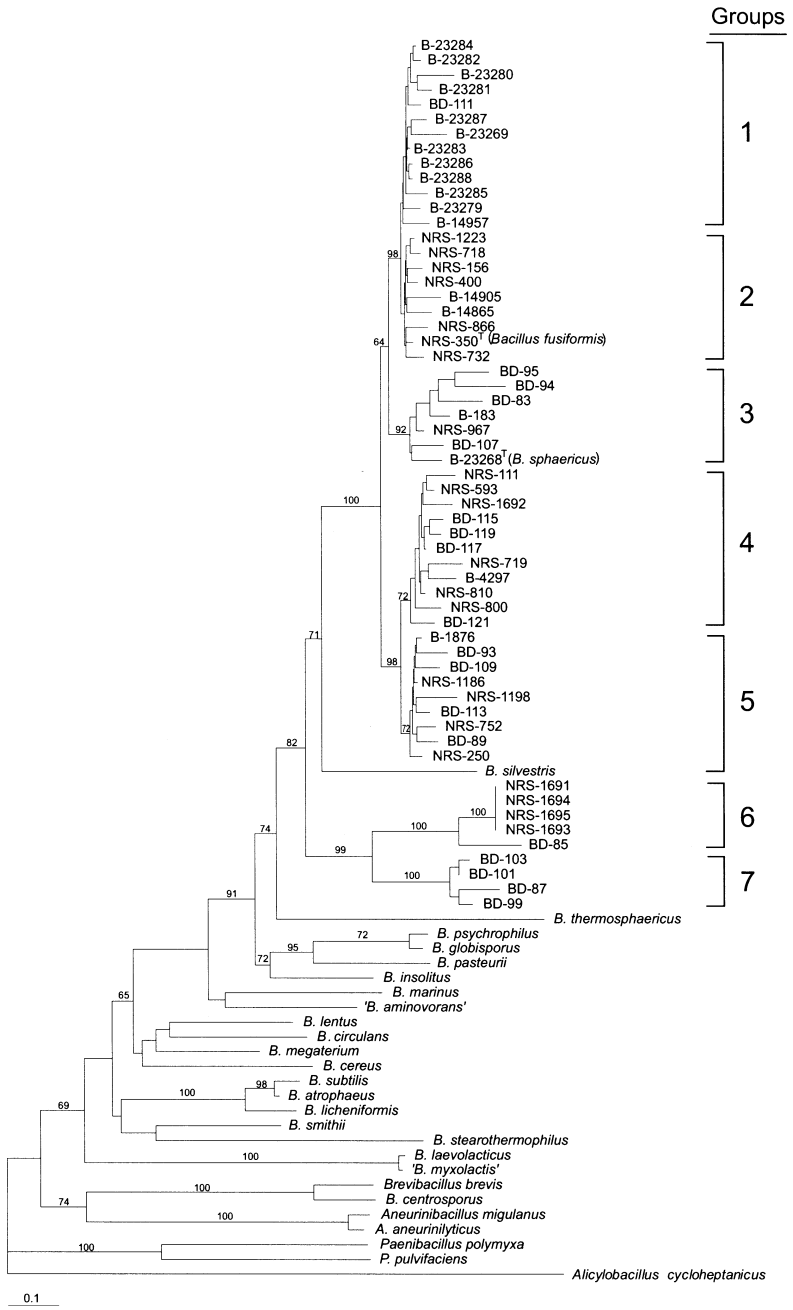

Fig. 1. Neighbour-joining tree showing the phylogenetic relationships among the *Bacillus sphaericus*-like strains, other round-spored *Bacillus* species, and principal rRNA *Bacillus* groups. The analysis is based on 1321 nucleotide sequences. Confidence limits estimated from bootstrap analyses (500 replications) appear at the nodes. A maximum-parsimony tree generated from the sequence data exhibited similar topology to this tree. *Alicyclobacillus cycloheptanicus* was designated as the outgroup species for the analyses. Bar, 1 nucleotide substitution per 100 nucleotides. Nucleotide sequences obtained from GenBank and used in the phylogenetic analyses were as follows: *Alicyclobacillus cycloheptanicus* 1457 (X51928), *Aneurinobacillus aneurinolyticus* NCIMB 10056 (X60645), *Aneurinobacillus migulanus* ATCC 9999^T (D78462), '*Bacillus aminovorans*' NCIMB 8292 (X62178), *Bacillus atrophaeus* NCIMB 12899^T (X60607), *Bacillus cereus* NCTC 11143 (X55063), *Bacillus circulans* IAM 12462^T (D78312), *Bacillus globisporus* DSM 4^T (X68415), *Bacillus insolitus* DSM 5^T (X60642), *Bacillus laevolacticus* IAM 12322 (D16269), *Bacillus lentus* IAM 12466^T (D16272), *Bacillus licheniformis* DSM 13^T (X68416), *Bacillus megaterium* IAM 13418^T (D16273), *Bacillus myxolacticus* IAM 12326 (D16274), *Bacillus pasteurii* NCIMB 8841 (X60631), *Bacillus psychrophilus* ATCC 23304^T (X60634), *Bacillus smithii* DSM 4216^T (X60643), *Bacillus stearothermophilus* strain T10 (X57309), *Bacillus subtilis* NCDO 1769 (X60646), *Bacillus thermosphaericus* strain P-11 (X90640), *Brevibacillus brevis* JCM 2503^T (D78457), *Brevibacillus centrosporus* NRRL NRS-664^T (D78458), *Paenibacillus polymyxa* NCDO 1774 (X60632) and *Paenibacillus pulvifaciens* NCDO 1141 (X60036).

incubated at 28 °C on nutrient agar amended with 5 mg $MnSO_4 . H_2O$ l^{-1} until sporulation occurred and then were stored at 4 °C.

Characterization. The method of Gordon *et al.* (1973) was used for characterizing the strains biochemically and physiologically. Because mainly negative reactions were obtained with this method, the BIOLOG GP characterization system (BIOLOG) was used to analyse an extended array of metabolic traits of a representative number of strains. Whole-cell fatty acid profiles were determined using the MIDI system of Sasser (1990). For this determination, the organisms were grown on TSBA for 24 h at 28 °C; triplicate plates were prepared to obtain sufficient cell mass for the assay.

The data obtained by the BIOLOG GP system were used to determine the extent of phenotypic similarity among the strains. Reactions were read after 24 h incubation at 28 °C. Similarity was estimated by means of the simple matching coefficient (S_{SM}) and clustering was based on the unweighted pair group arithmetic average-linkage algorithm (Sneath & Sokal, 1973). A matrix consisting of 30 substrates and 51strains was used for the computation.

16S rRNA gene sequencing. 16S rRNA gene fragments corresponding to positions 9–1510 of *Escherichia coli* were amplified by the PCR using purified DNA and primers 27f and 1492r (Lane, 1991). Amplification products were purified with a GeneClean II Kit (Bio101) and were sequenced with a Prism ABI dideoxy terminator cycle sequencing kit (Applied Biosystems). The protocols used were those recommended by the manufacturers. Sequences were determined with an automated DNA sequencer (model 377; Applied Biosystems). Both strands were sequenced using the primers 27f, 530f, 801f, 1114f, 1406f, 109r1, 342r, 685r3, 907r, 1100r and 1492r (Lane, 1991; Nakamura, 1996). The CLUSTAL V program (Higgins *et al.*, 1992) was used to align the 16S DNA sequences generated with sequences of selected members of the family *Bacillaceae* obtained from GenBank (Larsen *et al.*, 1993). Genetic distance was computed by using Kimura's two-parameter model (Kimura, 1980) and used for neighbour-joining analysis. Phylogenetic trees were constructed using neighbour-joining and maximum-parsimony methods provided by PAUP 4.0b1 (D. L. Swofford, unpublished); both methods produced trees with similar topologies. Nucleotide sequences generated in this study have been deposited with GenBank under the accession numbers shown in Table 1.

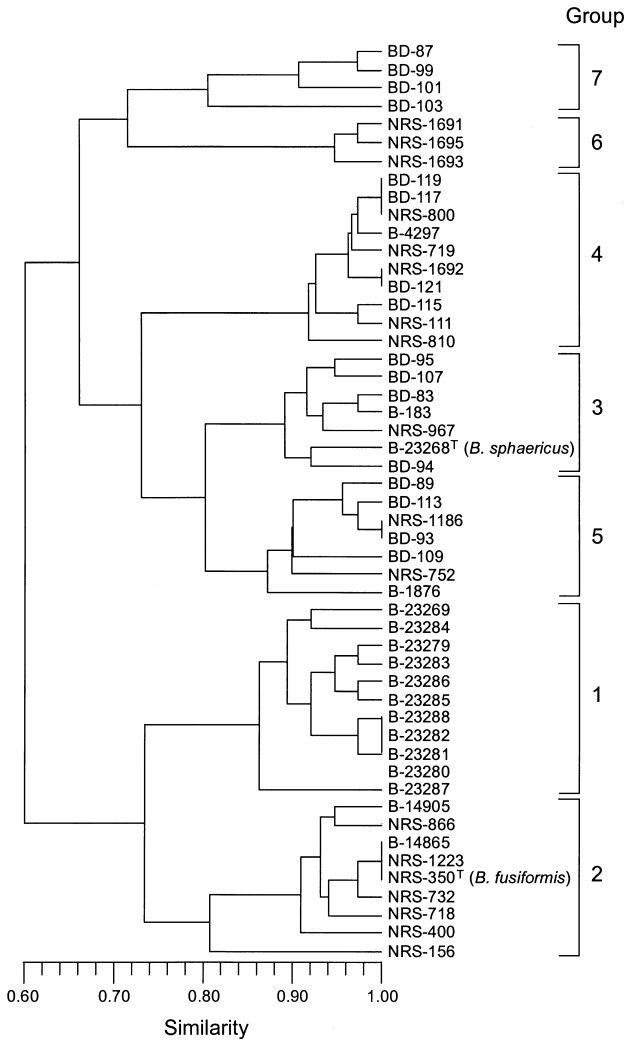

Fig. 2. Dendrogram showing relationships among the *B. sphaericus*-like strains, based on substrate utilization in BIOLOG GP test plates. Similarity was estimated by means of the simple matching coefficient (S_{SM}) and clustering was based on the unweighted pair group arithmetic average-linkage algorithm (Sneath & Sokal, 1973). A matrix consisting of 30 substrates and 51 strains was used for the computation. Group numbers correspond to those in Fig. 1.

RESULTS

Fig. 1 presents a phylogenetic tree based on comparison of the 16S rDNA sequences generated in this study with sequences of representative *Bacillus* species, including the round-spored organisms, obtained from GenBank. The *B. sphaericus*-like organisms separate into seven groups (six of which are statistically well supported by bootstrap values of 70% or better) within rRNA group 2 of the genus *Bacillus* (Ash *et al.*, 1991). Two of the clades represent the recognized species *B. fusiformis* (group 2) and *B. sphaericus* (group 3); each group encompassed its respective type strain. As observed by others (Krych *et al.*, 1980; Alexander & Priest, 1990; Aquino de Muro *et al.*, 1992; Woodburn *et al.*, 1995; Rippere *et al.*, 1997), the mosquitocidal or insect-associated strains cluster in one clade (group 1) that is closely linked to *B. fusiformis*. The sequence similarity of group 1 and 2 strains averages approximately 99·1%. However, the separation of the two groups is not statistically well supported. Somewhat separated from these three clades are four others represented by 4–11 strains. A complex consisting of groups 4 and 5 shares a common lineage with the *B. fusiformis–B. sphaericus* complex. Groups 6 and 7 appear in a separate complex, as does *B. silvestris*. The presently recognized round-spored psychrophilic species *Bacillus globisporus*, *Bacillus psychrophilus*, *Bacillus insolitus*, *Bacillus marinus* and the alkalophile *Bacillus pasteurii* form a separate group distinct from the *B. sphaericus sensu lato* groups. '*Bacillus aminovorans*' and *Bacillus thermosphaericus* are distant outliers.

Table 2. Substrate-utilization patterns useful for differentiating the *B. sphaericus*-like groups

Numbers in parentheses indicate the numbers of strains studied. The numbers of positive reactions are shown. For groups 1 and 2, all 11 and 9 strains, respectively, were positive. L-Glutamate was used by all strains, 65 substrates were not used at all and variable reactions were obtained with 26 substrates.

Substrate*	Group				
	3 (7)	4 (10)	5 (7)	6 (3)	7 (4)
Pyruvate	7	10	7	3	0
L-Alanine	7	10	7	0	0
Glycyl-L-glutamate	7	10	7	0	4
2′-Deoxyadenosine	0	0	7	0	4
Inosine	7	0	7	0	4
AMP	0	0	0	0	4
UMP	0	0	0	0	4

*Substrates with which 0 or 100 % reactions were obtained.

The dendrogram in Fig. 2 shows the relationships of the *B. sphaericus*-like organisms, based on phenotypic characterization obtained using the BIOLOG system. A representative number of strains from each phylogenetic group were analysed. Analysis based on phenotypic traits segregates the organisms into seven groups that are similar to the 16S rDNA phylogenetic groups. These organisms do not metabolize the common hexoses, pentoses or disaccharides, but they prefer pyruvate, amino acids, purine or pyrimidine bases and related compounds as their energy and carbon sources. Table 2 shows the differentiation of the seven groups based on the oxidation patterns of these classes of substrates which include pyruvate, L-alanine, glycyl-L-glutamate, 2′-deoxyadenosine, inosine, AMP and UMP.

Whole-cell fatty acid profiles are also useful for group distinctions. Although all the groups contain the same compounds, significant differences occur in the amounts of seven acids, namely 14:0, 15:0, 15:0 iso, 15:0 anteiso, 16:0, 16:0 iso, 16:1 ω11*cis* fatty acids and 16:1 ω7*cis* alcohol (Table 3) and can be used for distinguishing among the groups. Other fatty acids observed but showing no significant compositional differences are 14:0 iso (1·86 %), iso 17:1 ω10*cis* (0·26 %), 17:0 iso (1·83 %) and 17:0 anteiso (0·59 %).

DISCUSSION

Although all the round-spored species share phylogenetic membership in *Bacillus* rRNA group 2 (Ash *et al.*, 1991), the mesophilic *B. sphaericus*-like species along with *B. silvestris* cluster apart from the psychrophilic, alkalophilic and thermophilic round-spored forms. Moreover, phylogenetic analysis shows that *B. sphaericus sensu lato* is a heterogeneous taxon consisting of at least seven genetically distinct groups. Group 2 represents *B. fusiformis* and group 3 represents *B. sphaericus*. Group 1 is closely related to group 2 and mainly encompasses the pathogenic strains; NRRL B-23280, NRRL B-23281, NRRL B-23288 and NRRL BD-111 are non-pathogenic members. On the basis of fatty acid analyses, Frachon *et al.* (1991) found that not all strains assignable to group 1 were pathogenic. Similarly, when comparing pathogens and non-pathogens, Rippere *et al.* (1997) found that not all organisms included in a DNA similarity group (corresponding to group 1 of this study) were pathogenic. The four remaining groups do not fit into any presently recognized round-spored species, namely *B. globisporus*, *B. insolitus*, *B. pasteurii*, *B. psychrophilus*, *B. silvestris*, *B. thermosphaericus* or '*B. aminovorans*'; they probably represent new species which are being further characterized.

Four of the phylogenetic groups correspond to the DNA relatedness groups reported by Krych *et al.* (1980). Group I of Krych *et al.* (1980) represents *B. sphaericus* and corresponds to group 3 of this study, group IIA to group 1, group IIB to group 2, and group III to group 4. Group IIA encompassed the pathogenic

Table 3. Fatty acid composition (means ± standard deviations) of the *B. sphaericus*-like organisms

Fatty acid	Fatty acid composition (%) of group:						
	1	2	3	4	5	6	7
14:0 iso	0·91 ± 0·01	2·57 ± 0·35	1·75 ± 1·29	1·92 ± 1·01	0·86 ± 0·27	ND	5·01 ± 1·20
15:0	1·61 ± 1·95	3·82 ± 0·03	1·47 ± 1·04	0·82 ± 1·15	2·02 ± 0·05	ND	1·65 ± 0·42
15:0 iso	53·01 ± 0·85	36·56 ± 1·62	44·46 ± 0·88	49·26 ± 0·23	22·83 ± 2·92	70·32 ± 0·59	23·58 ± 2·56
15:0 anteiso	7·85 ± 0·76	11·19 ± 0·76	11·93 ± 0·81	11·94 ± 0·98	8·21 ± 3·68	8·11 ± 1·15	17·80 ± 1·68
16:0	ND	2·53 ± 0·36	1·98 ± 1·40	1·87 ± 0·14	0·47 ± 0·66	ND	5·35 ± 0·49
16:1 ω7*cis* alcohol	13·85 ± 0·19	13·73 ± 4·81	12·75 ± 0·52	12·25 ± 0·86	19·97 ± 0·10	6·02 ± 1·53	4·74 ± 1·01
16:0 iso	10·63 ± 0·20	18·27 ± 8·70	12·75 ± 0·51	9·69 ± 0·83	27·46 ± 0·29	3·08 ± 0·18	8·06 ± 2·86
16:1 ω11*cis*	2·54 ± 0·52	3·68 ± 0·26	3·23 ± 0·56	3·81 ± 0·08	4·42 ± 0·25	1·41 ± 0·26	14·47 ± 1·62

ND, Not detected.

strains and group IIB encompassed strains of *B. fusiformis*. Some strains in the ungrouped strains of Krych *et al*. (1980) are in group 5 (B-1876, NRS-250, NRS-1186) and group 6 (NRS-1691, NRS-1695) of the present study.

High 16S rDNA sequence similarity of approximately 99·1 % and S_{SM} values of approximately 73 %, respectively, between groups 1 and 2 indicate a close relationship for the two groups. As noted in the present study, DNA relatedness of about 60 % (Krych *et al*., 1980), RAPD analyses (Woodburn *et al*., 1995) and RFLP-pattern assays (Aquino de Muro *et al*., 1992) demonstrated close enough relatedness between groups IIA and IIB to suggest that the pathogenic strains represented a subtaxon or 'sister' species of *B. fusiformis*. The absence of compelling differences between the groups has discouraged the proposal of a subspecies. With the *B. sphaericus*-like bacteria, the classical characterization methods (Gordon *et al*., 1973) failed to produce decisive taxonomic information. Using a broadened range of substrates, Alexander & Priest (1990) obtained data that permitted several groups within *B. sphaericus sensu lato* to be distinguished. In our study, the BIOLOG GP system yielded substrate utilization data that, when analysed numerically, supported the existence of seven groups among the *B. sphaericus*-like organisms examined and provided a set of substrates useful for differentiating several of the groups. Whole-cell fatty acid profiles provided further data supporting the presence and differentiation of the seven groups.

Comparison of the results from the present study with those of others (Krych *et al*., 1980; Aquino de Muro *et al*., 1992; Woodburn *et al*., 1995) has revealed several anomalies. In the present study, NRRL NRS-400 and NRRL NRS-1693 appear in the *B. fusiformis* clade and in an unnamed taxon (group 6), respectively. RFLP patterns suggested slight differences between these two strains (Aquino de Muro *et al*., 1992). In contrast, DNA similarity (Krych *et al*., 1980), numerical analysis (Alexander & Priest, 1990) and RAPD analysis (Woodburn *et al*., 1995) indicated that the two strains were members of the same group. In other examples, the results of our study clustered NRRL NRS-1223 and NRRL B-183 with *B. fusiformis* and *B. sphaericus*, respectively. However, these strains were assigned to group III (not *B. fusiformis* or *B. sphaericus*) and as miscellanea based on DNA-similarity analyses (Krych *et al*., 1980). In the present study, NRRL NRS-1198 is a member of group 5, which encompasses (among others) NRRL B-1876, NRRL NRS-250 and NRRL NRS-1186. Studies by Krych *et al*. (1980) and Woodburn *et al*. (1995) showed no similarity between NRRL NRS-1198 (a member of group V; Krych *et al*.) and the three strains mentioned (miscellanea; Krych *et al*.). The reasons for these discrepancies are not readily apparent. However, they can be resolved by retrieving the cultures from the various laboratories and carrying out DNA similarity and sequence studies.

Molecular biological and phenotypic studies have revealed the diversity of the round-spored species *B. sphaericus sensu lato*. It encompasses *B. sphaericus*, *B. fusiformis* and four possible new species, the creation of which would increase the list of nine extant species (*B. fusiformis*, *B. globisporus*, *B. insolitus*, *B. marinus*, *B. pasteurii*, *B. psychrophilus*, *B. silvestris*, *B. sphaericus* and *B. thermosphaericus*) to 13. The dependence of early studies on relatively insensitive characterization methods hindered detection of the diversity and fostered the creation of a heterogeneous round-spored mesophilic species that included both pathogens and non-pathogens of insects. Thus, pathogenic variability accrues from genetic variability and incorrect classification. Diversity also implies the existence of other undiscovered mesophilic round-spored species.

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