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Comparative 16S rRNA Oligonucleotide Analyses and Murein Types of Round-spore-forming Bacilli and Non-spore-forming Relatives

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The phylogenetic incoherency of the genus *Bacillus* as presently described is demonstrated by analysis of both published and new data from comparative 16S rRNA cataloguing of nine *Bacillus* species and a number of related non-*Bacillus* taxa, i.e. *Caryophanon latum*, *Filibacter limicola* and *Planococcus citreus*. While the ellipsoidal-spore-forming bacilli, e.g. *B. subtilis* and allied species, formed a coherent cluster, the round-spore-forming bacilli showed a higher degree of relationship to the non-spore-forming organisms than these bacilli show among each other. Thus *B. sphaericus* clustered with *C. latum*, *B. globisporus* grouped with *F. limicola*, *B. pasteurii* with *Sporosarcina ureae*, and '*B. aminovorans*' with *P. citreus*, respectively. These organisms formed two related subclusters which, in their phylogenetic depth, are comparable to that of the *B. subtilis* subline. With the exception of '*B. aminovorans*', the 16S rRNA phylogeny was entirely consistent with the distribution of murein types. Even more distantly related to and grouping outside the main *Bacillus* cluster was *B. stearothermophilus*, which displayed a moderate relationship to *Thermoactinomyces vulgaris*. Taxonomic problems arising from the new insights into the intrageneric relationships of *Bacillus* are discussed.

INTRODUCTION

Gram-positive, rod-shaped, spore-forming bacteria that are aerobic or facultatively anaerobic have traditionally been assigned to the genus *Bacillus*. The wide range of nutritional requirements, growth conditions and DNA base composition, as well as the metabolic diversity, underline the phenetic and genetic diversity of this genus (Fahmy *et al.*, 1985; Claus & Berkeley, 1986). DNA–DNA hybridization studies have indicated that most species are only distantly related (Seki *et al.*, 1978; Priest, 1981). Applying the 16S rRNA oligonucleotide cataloguing approach to a restricted number of bacilli, three clusters of moderate relationships were found: *B. subtilis*, *B. pumilus*, *B. megaterium* and *B. cereus* formed the first group; *B. pasteurii* and *Sporosarcina ureae* the second; and *B. stearothermophilus* the third (Fox *et al.*, 1977, 1980). The close relationship of *S. ureae* to bacilli, in particular *B. pasteurii*, has also been demonstrated by

Abbreviation: *meso*-A_2pm, *meso*-diaminopimelic acid.

DNA–RNA hybridization studies (Herndon & Bott, 1977). Besides *S. ureae*, other spore-forming organisms, such as *Sporolactobacillus inulinus* (Fox *et al.*, 1977) and *Thermoactinomyces vulgaris* (Stackebrandt & Woese, 1981*a*), as well as some non-spore-forming organisms, such as *Planococcus citreus* (Stackebrandt & Woese, 1979) and *Filibacter limicola* (Clausen *et al.*, 1985) were found to cluster phylogenetically with bacilli. In contrast to most bacilli, which contain *meso*-diaminopimelic acid (*meso*-A_2pm) as diamino acid in their murein (Schleifer & Kandler, 1972), the two asporogenous organisms contain lysine (Schleifer & Kandler, 1972; Clausen *et al.*, 1985). In this respect, these asporogenous organisms resemble the round-spore-forming bacilli, namely *B. pasteurii*, *B. sphaericus* and *S. ureae* (Ranftl & Kandler, 1973; Schleifer & Kandler, 1972). In the present study, we have determined the 16S rRNA oligonucleotide catalogues and murein types for several additional round-spore-forming bacilli and the asporogenous trichome-forming *Caryophanon latum* (Trentini, 1986) in order to obtain further insight into the phylogenetic and phenotypic relationships among members of the genus *Bacillus* and their asporogenous relatives.

METHODS

B. subtilis strain 168, *B. megaterium* KM-A290, *B. cereus* strain M. Starr, *B. pumilus* strain Illinois, *B. pasteurii* ATCC 11859 and *B. stearothermophilus* ATCC 12978 were used; *Sporosarcina ureae*, *Planococcus citreus* and *Filibacter limicola* were the same cultures as described previously (Fox *et al.*, 1977; Stackebrandt & Woese, 1979; Clausen *et al.*, 1985). '*Bacillus aminovorans*' DSM 1314 was grown in CASO-bouillon (Merck), whereas *B. sphaericus* ATCC 14577, '*B. psychrophilus*' (*B. globisporus* ATCC 23304) and *B. insolitus* ATCC 23299 were grown according to Pechman *et al.* (1976). All strains were grown aerobically, at 30 °C in the case of '*B. aminovorans*' and *B. sphaericus*, and at 20 °C for the other *Bacillus* isolates. *Caryophanon latum* strain L (R. Naeveke, Technical University, Braunschweig, FRG) was grown aerobically at 25 °C on agar plates (0·2%, w/v, yeast extract; 0·5%, w/v, bacteriological peptone; 0·5%, w/v, sodium acetate; 1·2%, w/v, agar in 10 mM-Tris/HCl buffer; pH 7·6). Labelling and sequence analysis of 16S rRNA followed either of the two standard procedures (Uchida *et al.*, 1974; Woese *et al.*, 1976; or Stackebrandt *et al.*, 1985). S_{AB} values were calculated as described by Fox *et al.* (1977). Murein types were determined according to Schleifer & Kandler (1972).

RESULTS AND DISCUSSION

In an initial survey on the phylogeny of six *Bacillus* species, *Sporosarcina ureae* and *Sporolactobacillus inulinus* (Fox *et al.*, 1977), 34 universal oligonucleotides ($\geqslant$hexamers) were listed as occurring in each of the 16S rRNA catalogues. With few exceptions, these sequences were also present in the catalogues of the five strains investigated in this study. These exceptions are AAUUCCACG, which is missing in *B. insolitus*, *B. sphaericus* and *C. latum* and AACACCAG, which is missing in *B. globisporus*. In addition the reader should note that the correct 3′ terminal oligonucleotide is $AUCACCUCCUUUCU_{OH}$ in all cases. Table 1 records the remaining oligonucleotides of the new 16S rRNA catalogues. Table 2 shows the individual binary comparisons (S_{AB} values) for these organisms. together with the values obtained with strains whose membership to the enlarged *Bacillus* cluster has already been demonstrated (Pechman *et al.*, 1976; Fox *et al.*, 1977; Stackebrandt & Woese, 1979, 1981*a*; Clausen *et al.*, 1984). The range of S_{AB} values obtained for the *Bacillus* strains analysed here (0·60–0·70) is essentially the same as those found for the bacilli investigated earlier by Fox *et al.* (1977). *C. latum*, not considered to be related to members of *Bacillus* in phenetic studies, shares a higher value (0·70) with *B. sphaericus* than the latter species shares with other bacilli. Even more pronounced is the relationship between '*B. aminovorans*' and *P. citreus* on the one hand, and the former species and *B. insolitus* on the other (0·75 and 0·73, respectively).

An average linkage dendrogram (Fig. 1) shows that all bacilli, except *B. stearothermophilus*, clustered together to form a side branch of the subdivision of Gram-positive eubacteria, defined by a low G + C content (<55 mol%). With the inclusion of the non-spore-forming species *C. latum*, this cluster was intermixed with strains that do not match the minimal description of *Bacillus*, i.e. the non-spore-forming, rod-shaped *F. limicola*, the spherical *P. citreus*, and the spore-forming but non-rod-shaped *S. ureae*. *B. stearothermophilus* exhibited a remote but distinct relatedness to *Thermoactinomyces vulgaris*, and together they branched off slightly earlier from the bulk of the bacilli. *Sporolactobacillus* (*Spl.*) *inulinus* was the only *Bacillus*-type organism

Table 1. *Partial oligonucleotide catalogues of 'B. aminovorans', B. insolitus, B. globisporus, B. sphaericus and C. latum*

Sequences found to be universal for *Bacillus* and related taxa (Fox *et al.*, 1977) are omitted. Likely sequence locations of the oligomers as compared to the known *B. subtilis* 16S rRNA sequence are indicated when these can be deduced. 1, '*B. aminovorans*'; 2, *B. insolitus*; 3, *B. globisporus*; 4, *B. sphaericus*; 5, *C. latum*.

Sequence	Organisms	Position
6-mers		
AAAAUG	1	–
AACAAG	1, 2, 4	456
AACACG	3	–
AACCAG	1	–
AACUUG	3, 5	635
AAUAAG	5	456
AAUCUG	5	–
ACAACG	1, 4	–
ACAUAG	5	1002
ACACUG	4, 5	740
ACAUCG	5	982
AUAAAG	4, 5	416
AUACAG	3	–
AUUUAG	2	–
AUUUCG	4, 5	423
CCCACG	1, 3, 4	617
CCCCCG	5	617
CUAACG	1–5	850
CUAUAG	4, 5	–
UAACUG	1–5	734
UCACUG	1	–
UCACUG	3	1142
UCCACG	1–5	791
UUUCCG	1–5	828
UUUUCG	2, 3	423
7-mers		
AUACAAG	1	–
AUCUUAG	1–4	1106
AUUUUAG	3	91
CAAACAG	1–5	766
CAACCUG	1, 3	124
CACUUUG	1	–
CAUCUCG	1	1304
CAUUAAG	2, 3	856
CAUUCAG	1–5	1120
CAUUUAG	1	–
CUCUCUG	3	722
CUUUCUG	3	–
UACAAAG	1	1234
UACCUUG	1, 2, 4	481
UAUUAUG	2, 3	–
UCAAAUG	1	–
UCCUUAG	5	–
UUAAAAG	2	–
UUCUCAG	2, 4, 5	1281
UUUUAUG	4	–
8-mers		
ACAAACCG	1, 4, 5	1154
ACAUCCCG	1, 4, 5	982
ACUAUCUG	4	721
ACUUUCUG	2	721
AUAAACCG	2, 3	1154
AUCUAAAG	1	749
AUUCUCCG	1	721
CAACCCCG	2	400
CACACUCG	4	–
CACUCUCG	5	1132
CCAACCCG	3, 5	–
CCAAUCCG	5	1264
8-mers (continued)		
CCCCUCAG	1, 5	834
CCCCUUAG	2–4	834
CCCUACAG	3	–
CCUACAUG	2, 4, 5	295
CCUCCAUG	3	–
CUAAUCCG	4	1264
CUCAACCG	1–5	624
CUCCUUUG	4	–
UACCUUUG	5	462
UUACCUUG	5	480
9-mers		
AAUAAUCAG	3	–
AAUCUUCCG	1	368
AAUUCCAAG	2, 5	661
AAUUUUUAG	5	–
ACAAUACUG	4	992
ACUUCAUCG	5	–
AUAACUCCG	1–5	145
AUACAAACG	4	–
AUUUCAUUG	1	942
AUUUCUUCG	5	210
CAACACCCG	1	1411
CAACCCUUG	1–5	1097
CACUCUAAG	1–4	1132
CAUCCUACG	1	343
CCCUUCAUG	4	–
CCUACCAAG	3	272
CUACAAACG	5	–
CUCACCAAG	1, 2, 4, 5	272
CUCCUUUCG	1	1057
CUUCUUCUG	2	983
UAACACCCG	2, 3, 5	1411
UCACUACAG	2	1142
UCCUUUAAG	2	595
UCUUUUAAG	1	595
UUCUUUAAG	3, 5	595
UUUCUUAAG	4	595
⩾10-mers		
AAAUCCCACG	2	613
AAUAAUAUCG	1	164
ACAAACUCUG	3	–
UAAAACUCUG	1, 2, 4, 5	434
ACAUCCCACUG	2, 3	–
CCAUCAUUUAG	4, 5	116
CCAUCCCACUG	5	–
UAACCCUUACG	1–3	1432
UACCUCAUUAG	2	492
UACCUUAUUAG	3–5	492
AAUAAUCUUUUG	4	–
CCUUUCCCUUCG	3	1009
UAACCCUUUUAG	5	–
AUCUUUCCCUUCG	2	1008
AACCUACCCUAUAG	4	125
AAUCUUCCACAAUG	2, 4, 5	368
CCAAUCCCAUAAAAG	1	1264
UAACCUACCUUAUAG	5	124
CUAAUCCCAUAAAACCG	2	1264
AAUAAUCCAUUUCCUCUCAUG	2	–
CCAAUCCCAUAAAAUCAUUCCCAG	3	1264

–, No likely position can be deduced.

Table 2. *Association coefficients for 'B. aminovorans', B. insolitus, B. globisporus, B. sphaericus and C. latum and a variety of bacilli and relatives whose 16S rRNA catalogues have been published (Pechman et al., 1976; Fox et al., 1977; Stackebrandt & Woese, 1979, 1981a; Clausen et al., 1985)*

	1	2	3	4	5
1 '*B. aminovorans*' DSM 1314	X				
2 *B. insolitus* ATCC 23299	0·64	X			
3 *B. globisporus* ATCC 23304	0·63	0·68	X		
4 *B. sphaericus* ATCC 14577	0·66	0·68	0·61	X	
5 *C. latum* L, Naeveke	0·59	0·62	0·59	0·68	X
B. subtilis strain 168	0·61	0·63	0·60	0·62	0·56
B. pumilus strain B. J. Lewis	0·63	0·65	0·63	0·65	0·60
B. megaterium KM-290	0·68	0·65	0·63	0·67	0·59
B. cereus strain M. P. Starr	0·63	0·64	0·59	0·65	0·59
B. pasteurii ATCC 11859	0·63	0·69	0·66	0·63	0·57
B. stearothermophilus ATCC 12978	0·63	0·55	0·59	0·56	0·53
S. ureae ATCC 6473	0·60	0·64	0·64	0·63	0·57
P. citreus ATCC 14404	0·74	0·74	0·69	0·70	0·63
F. limicola NCIB 11923	0·59	0·66	0·68	0·60	0·56
T. vulgaris strain P12	0·57	0·52	0·52	0·56	0·53
Spl. inulinus ATCC 15538	0·51	0·49	0·55	0·48	0·44

Table 3. *DNA base composition and murein type of round-spore-forming bacilli and related non-spore-forming bacteria*

Species	Strain	DNA mol% G + C	Murein type
'*B. aminovorans*'	DSM 1313	41	*meso*-A_2pm-direct
B. globisporus	DSM 3	40*	Lys-D-Glu
B. globisporus	DSM 4	40*	Lys-D-Glu
B. insolitus	DSM 5	36*	Orn-D-Glu
B. pasteurii	DSM 33	39*	Lys-Ala-D-Asp¶
B. sphaericus	DSM 28	37*	Lys-D-Asp**
C. latum	L, Naeveke	44–46†	Lys-D-Glu
F. limicola	NCIB 11923	44‡	Lys-D-Glu
P. citreus	DSM 20549	51§	Lys-D-Glu
S. ureae	DSM 2281	40‖	Lys-Gly-D-Glu

* Fahmy *et al.* (1985). † Trentini (1986). ‡ Maiden & Jones (1984). § Bohacek *et al.* (1967). ‖ Bohacek *et al.* (1968). ¶ Ranftl & Kandler (1973). ** Schleifer & Kandler (1972).

possessing a phylogenetic position different from that of other aerobic or facultatively anaerobic Gram-positive spore formers.

In the dendrogram presented here, it is assumed that all the 16S rRNAs included in the analysis are isochronic. As pointed out by Stackebrandt & Woese (1981*b*) the majority of *Bacillus* species and related taxa (*Sporosarcina*) are isochronic. Likewise, this holds for the non-*Bacillus* taxa grouping within the *Bacillus* cluster (*Filibacter*, *Thermoactinomyces*, *Planococcus*) and the strains investigated in this study. The few exceptions are *B. acidocaldarius* and *B. coagulans* (C. R. Woese unpublished data) and *Spl. inulinus*. Since organisms with a fast 'clock' exhibit lower S_{AB} values than isochronic strains, the branching point of *Spl. inulinus* must be higher than indicated by the S_{AB} value as depicted in the dendrogram. With proper correction for this non-isochronic behaviour of its rRNA, *Spl. inulinus* would cluster closer to the primary *Bacillus* group.

The intrageneric clustering of genuine *Bacillus* species separated strains harbouring terminally positioned spherical spores (*B. sphaericus*, *B. pasteurii* and *B. globisporus*) from those with centrally positioned ellipsoidal spores (*B. cereus*, *B. megaterium*, *B. pumilus* and *B. subtilis*). '*B. aminovorans*', defined by spherical spores positioned centrally, was recovered between these two clusters. This arrangement accords with the distribution of the murein type (Table 3), which

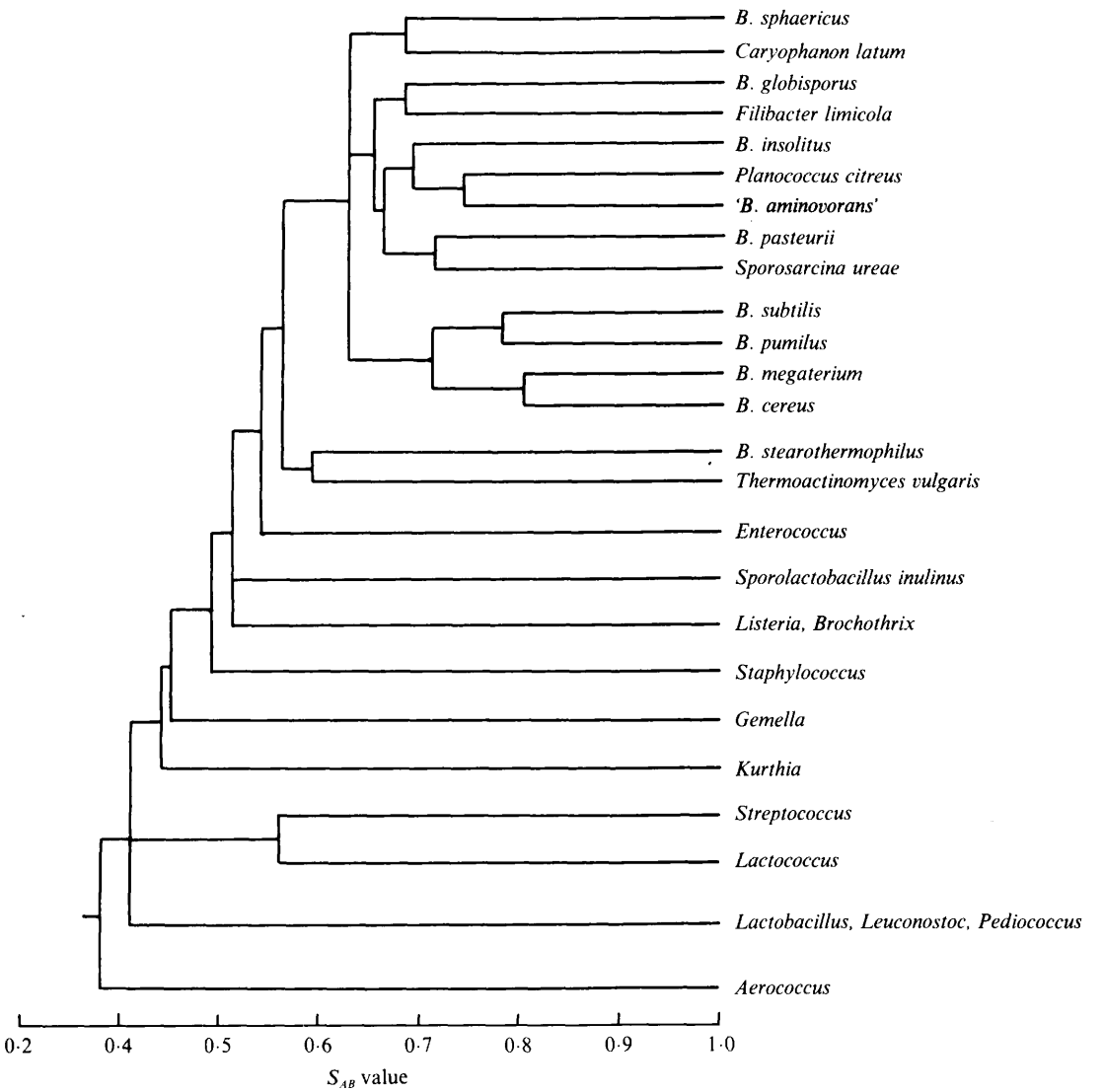

Fig. 1. Dendrogram of relationships displaying the phylogenetic position of bacilli and their spore-forming and non-spore-forming relatives.

groups '*B. aminovorans*' with the *meso*-A_2pm type (*B. subtilis* and relatives), distinct from those containing either lysine or ornithine in their murein. Conversely, the distribution of the DNA base composition only partially follows this pattern. Closely related strains of the first *Bacillus* group, i.e. *B. megaterium*, *B. cereus*, *B. pumilus* and *B. subtilis*, have the same value of about 36 and 44 mol%, respectively, while values ranging from 38–41 mol% are found in members of the second group, and in '*B. aminovorans*'.

With the inclusion of the non-spore-forming taxa with the genuine bacilli, the distribution of phenotypic characters is even more confused, not only in terms of morphological features but also in the mol% G + C of their DNA, and in the murein types. This is especially true for the phylogenetically closely related pair *P. citreus* and '*B. aminovorans*', which differ from each other in the diamino acid of their murein and by almost 10% DNA G + C content (Table 3).

The group of organisms investigated here presents a challenge to bacterial systematics. Initially, the situation is likely to further divide taxonomists into two opposing groups by giving

each of them arguments to defend their position. One school, by placing emphasis on similarities at the genotypic level, would favour either an emendation of a genetically defined genus *Bacillus* (which would, in addition, harbour the non-spore-forming relatives), or, a less straightforward step, the separation of the enlarged *Bacillus* cluster into several individual genera. In this case, one could think about a family *Bacillaceae*, embracing a redefined genus *Bacillus* (*B. subtilis* and relatives), the non-spore-forming relatives (*Filibacter* and *Caryophanon*) and a number of new genera, harbouring those bacilli not belonging to *Bacillus sensu stricto* (namely *B. globisporus*, *B. pasteurii* and others). This strategy takes into account the genetic relatedness among true bacilli and their relatives. Phenotypic characters used so far for the description of the round-spore-forming bacilli, together with information on the murein type, could then be used for the definition of individual genera. As also encountered in other regions the phylogenetic tree, e.g. rhodobacteria (Stackebrandt & Woese, 1984) or actinomycetes (Stackebrandt & Schleifer, 1984), and especially seen with aerobic organisms, many genetically moderately related organisms exhibit morphological similarities, which until recently had been used to cluster them in the same genus. Conversely, as verified in the order Actinomycetales, a number of families embrace morphologically and biochemically very different genera.

The approach of the other school would be to conceal phylogenetic relationships in order to obtain a simple identification and classification scheme. The presence of spores, a Gram-positive staining reaction, rod-shaped morphology and aerobic metabolism are characters easy to determine, which together would unambiguously allow an organism to be defined as a member of the genus *Bacillus*, irrespective of its phylogeny. The asporogenous relatives of *Bacillus* are readily characterized and their phenotypic features have never caused confusion with the description of a *Bacillus* species. Thus, for purely practical reasons, this school would argue that the present taxonomic status of *Bacillus* should be conserved. In this context it is important to raise the question of how to deal with sporeless mutants. Are they considered to be *Bacillus* strains (because their origin is known) or do they have to be described as members of a new genus? In the traditional concept, these organisms may be misidentified as members of different genera, and may not be considered to be relatives of bacilli at all; whereas in a phylogenetically/chemotaxonomically orientated classification, these strains would be grouped with *Bacillus* and related taxa at the genus level.

The phenotypic incoherency of the genus *Bacillus* has been recognized for many years (see Claus & Berkeley, 1986) and even the phylogenetic heterogeneity has been known for almost a decade (Fox *et al.*, 1977; Stackebrandt & Woese, 1979). Although the splitting of the genus *Bacillus* into two or more genera would provide a more 'natural' classification of these organisms, the decision to do it now would be premature. *Bergey's Manual of Systematic Bacteriology* (Claus & Berkeley, 1986) lists 34 validly described species of which only nine have been analysed by 16S rRNA cataloguing or comparative methods. It can be assumed that with more species investigated by methods measuring remote relationships, the phylogeny of members of the genus *Bacillus* might point towards an even greater degree of incoherency than expected and tolerated for a genus. In order to avoid confusion, such studies should be awaited before a formal dissection of the genus *Bacillus* with consequent description of new genera is proposed.

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