Applications and Systematics of *Bacillus* and Relatives

Edited by

Roger Berkeley
University of Bristol
Bristol
England

Marc Heyndrickx
Centrum voor Landbouwkundig
Melle
Belgium

Niall Logan
Glasgow Caledonian University
Glasgow
Scotland

Paul De Vos
Laboratorium voor Microbiologie
Gent
Belgium

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Dedicated to
Ruth Gordon

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a Blackwell Publishing company

350 Main Street, Malden, MA 02148-5018, USA
108 Cowley Road, Oxford OX4 1JF, UK
550 Swanston Street, Carlton, Victoria 3053, Australia
Kurfürstendamm 57, 10707 Berlin, Germany

First published 2002

Library of Congress Cataloging-in-Publication Data

Applications and systems of bacillus and relatives/
Roger Berkeley . . . [et al.].
p. cm.
Includes bibliographical references and index.
ISBN 0-632-05758-0 (hb: alk. paper)
1. Bacillus (Bacteria) I. Berkeley, Roger.
QR82.B3 A66 2002
579.3′62—dc21
2002022135

A catalogue record for this title is available from the British Library.

Set in 9.5/12pt Sabon
by Graphicraft Limited, Hong Kong
Printed and bound in the United Kingdom
by MPG Books Ltd, Bodmin, Cornwall

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List of Contributors

D.R. Arahal, *Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Seville, Spain*

D.J. Beecher, *Hazardous Materials Response Unit, FI Academy, Quantico, VA 22135, USA*

R.C.W. Berkeley, *Badock Hall, University of Bristol, Stoke Park Road, Bristol BS9 1JQ, England*

A. Bishop, *School of Chemical and Life Sciences, University of Greenwich, Wellington Street, Woolwich, London SE18 6PF, England*

C.P. Chanway, *Faculties of Forestry and Agricultural Sciences, University of British Columbia, Vancouver, British Columbia, Canada V6T 1Z4*

P. De Vos, *Laboratorium voor Microbiologie, Universiteit Gent, K L Ledgeganckstraat 35, B-9000 Gent, Belgium*

D. Fritze, *DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany*

A. Gambacorta, *Institute of Chimica Biomolecolare of CNR, Comprensorio Olivetti isol 70, Via Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy*

R. Goodacre, *Institute of Biological Sciences, University of Wales, Aberystwyth, Ceredigion SY23 3DD, Wales*

P.E. Granum, *Department of Pharmacology, Microbiology and Food Hygiene, Norwegian Veterinary College of Veterinary Medicine, PO Box 8146 Dep, N-0033 Oslo, Norway*

M. Heyndrickx, *Centrum voor Landbouwkundig Ondorzoek Gent, DVK, Bursselsesteenweg 370, B-9090 Melle, Belgium*

K.K. Hill, *Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA*

P.J. Jackson, *Bioscience Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA*

S.T. Jørgensen, *Novozymes A/S, Novo Alle, DK-2880 Bagsvaerd, Denmark*

P. Kämpfer, *Institut fur Angewandte Mikrobiologie, Fachbereig Agrarwissenschaften und Unweltsicherung, Uustus-Liebig-Universiteit Giessen, Heinrich-Buff-Ring 26–32, D-35392 Giessen, Germany*

P. Keim, *Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ 86011, USA*

A.-B. Kolstø, *Biotechnology Centre of Oslo and Institute of Pharmacy, University of Oslo, PB 1125 Blindern, 0316 Oslo, Norway*

L. Lama, *Institute of Chimica Biomolecolare of CNR, Comprensorio Olivetti isol 70, Via Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy*

N.A. Logan, *School of Biological and Biomedical Sciences, Glasgow Caledonian University, City Campus, Cowcaddens Road, Glasgow G4 0BA, Scotland*

J.T. Magee, *Department of Medical Microbiology and Public Health Laboratory, University Hospital of Wales, Heath Park, Cardiff CF4 4XW, Wales*

J. Mahillon, *Laboratorie de Genetique Microbienne UCL, Place Croix du Sud 2/12, B-1348 Louvain-la-Neuve, Belgium*

B. Nicolaus, *Institute of Chimica Biomolecolare of CNR, Comprensorio Olivetti isol 70, Via Campi Flegrei 34, 80078 Pozzuoli, Napoli, Italy*

J.R. Norris, *Langlands, 10 Langley Road, Bingley, West Yorkshire BD16 4AB, England*

H. Outtrup, *Skovhaven 89, DK-3500 Vaerloese, Denmark*

F.G. Priest, *Department of Biological Sciences, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, Scotland*

P. Scheldeman, *Centrum voor Landbouwkundig Onderzoek Gent, DVK, Brusselsesteenweg 370, B-9090 Melle, Belgium*

E. Stackebrandt, *DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany*

J. Swiderski, *DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany*

P.C.B. Turnbull, *Arjemptur Technology Ltd, Science Park, DSTL, Porton Down, Salisbury SP4 0JQ, England*

J. Van Rie, *Aventis Crop Science, Plateaustraat 22, B-9000 Gent, Belgium*

A. Ventosa, *Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Seville, Seville, Spain*

Foreword

In 1979, in Cambridge, the Systematics Group of the Society for General Microbiology held a meeting on the systematics of the aerobic, endospore-forming bacteria, and in 1981 a book based upon it was published by the SGM in its Special Publications series. That book, *The Aerobic Endospore-forming Bacteria: Classification and Identification*, was edited by Roger Berkeley and Mike Goodfellow, and for a number of years it served as a valuable reference work in the field, and was widely cited in publications dealing with *Bacillus* species.

All the contributors to the Cambridge meeting were well aware that the *Approved Lists of Bacterial Names* were soon to be published, and indeed these appeared in the following year. In it, the number of valid *Bacillus* species was reduced to 31, reflecting a considerable tidying up of the genus.

By 1997, when planning for the meeting on which the present volume is based began, members of the genus *Bacillus* had been allocated to six genera, with a total of about 140 species. This progress was largely driven by the application of sophisticated chemotaxonomic and genetic characterization methods, and the use of powerful computers to analyse the resulting data. Indeed, it was this explosion in species numbers, the pace of change in the taxonomy of the aerobic endospore-forming bacteria, and the absence of any comprehensive and up-to-date treatment of the systematics of the group, that suggested the idea for the '*Bacillus* 2000' meeting.

The background of the meeting was thus a desire to bring taxonomists interested in *Bacillus* and its relatives together with those who use or combat these organisms in medicine, agriculture, food and industry. The meeting was held in Bruges (Belgium) in August 2000, and one measure of its success was the number of people who at its end agreed that they found it difficult to remember when they last enjoyed hearing every paper in each session from its brief introduction to the concluding remarks. We therefore warmly thank all those who contributed to the meeting, the poster display and this book.

The organizers of the meeting and editors of this book also acknowledge with gratitude the financial support from FEMS, without which the whole enterprise would have been impossible. Important financial contributions were also made by the Belgian Society for Microbiology and by a number of commercial organizations (bioMérieux, Applied Maths, Belgian Coordinated Collection of Micro-organisms, B. Braun Biotech International, Van Hopplynus, Bio-Rad Laboratories, *P.E.* Biosystems and MERCK Eurolab), and we are most grateful to all of them too. We also acknowledge the University of Ghent for its practical

and material support in the organization of the poster session. Finally, we wish to thank our secretarial colleagues for their invaluable assistance.

Another, and most startling, feature of the meeting was that, on the evening of the day before it began, one conference member asked 'When is the next meeting like this going to take place?'! We strongly believe that the quality of the meeting lived up to the expectation implied in that question, and initial arrangements are being made for the next meeting, probably to be held in Slovenia in the summer of 2003.

Roger Berkeley
Marc Heyndrickx
Niall Logan
Paul De Vos

Chapter 1

Whither *Bacillus*?

Roger C.W. Berkeley

Introduction

'Whither . . . – To what place, position; what is the future of.' (*The Concise Oxford Dictionary,* Sixth edition.)

The beginning of a new millennium is a major historical milestone and it is appropriate to look at what has happened recently to the genus *Bacillus* and what might happen to it in the future. But first, to give a proper perspective, this should be preceded by a glance into the past.

The history of the genus *Bacillus* is long, and interwoven with the early history of bacteriology. '*Vibrio subtilis*' – now *Bacillus subtilis* – was described in 1835 by Ehrenberg and in 1864 Davaine allocated the name '*Bacteridium*' to the organism associated with anthrax. But it was Cohn, in 1872, who proposed the genus *Bacillus*. All this happened before the final resolution of the debate about whether spontaneous generation occurred or not!

In the 130 years since the creation of this genus its systematics have, unsurprisingly, undergone massive changes. Those up to 1979 were reviewed by Gordon (1981), and a numerical summary from her chapter, listing the number of species assigned to *Bacillus* in each of the first eight editions of *Bergey's Manual of Determinative Bacteriology* (table 1.1), gives a flavour of changes in the 50 years or so spanning the middle of the last century.

In her review, Ruth Gordon remarked that the (large) number of species assigned to the genus in the first to the fifth editions of the *Manual* make it obvious that many new species were named and described without, using the words of Cowan and Steel (1974), '. . . the comparative work necessary to put an organism into its rightful place in an existing genus or species'. Ruth's standards of comparative work were high. The work in which she was involved was based initially on a collection of 621 strains, later expanded to 1134 strains. Furthermore, as I heard her explain in her characteristically simple, quiet and modest way, at the very beginning of my scientific career, to a meeting of the Society for General Microbiology in London: if a colony of appearance different to the majority appeared on a plate, it was not assumed that the culture was contaminated. Instead, attempts would be made to isolate the organism with the different colonial morphology and to study it until it was certainly established that it really was a contaminant and not a variant. In this way, and by studying the limits of variability for some species, she and her colleagues were able to eliminate

Table 1.1 Numbers of species assigned to the genus *Bacillus* in different editions of *Bergey's Manual* up to 1974 (modified from Gordon 1981).

Bergey's Manual	Year	Number of species
1st edition	1923	75
2nd edition	1925	75
3rd edition	1930	93
4th edition	1934	95
5th edition	1938	146
6th edition	1948	33
7th edition	1954	25
8th edition	1974	Group I: 22 Group II: 26

some species and to demote others to lesser rank. Thus, the 146 species in the 5th edition of *Bergey's Manual* were reduced to 33 in the 6th edition and to 25 in the 7th.

In the 8th edition there was a further reduction in the number of species. These fell into two groups. In Group I there were 22 which were widely accepted as distinct entities, whereas the 26 in Group II had received less widespread recognition (table 1.1). An editorial note records that there was considerable correspondence between the *Manual*'s editors and the authors of the section on *Bacillus*, about the status of the species in Group II. This states that 'In many genera, most, if not all the species in this Group would have been listed as species *incertae sedis*, and one author agrees'. That person was certainly Ruth Gordon whose thorough, painstaking work is a model for us all. It resulted in a taxonomic arrangement of the genus *Bacillus* which largely 'worked' – although not without problems – for most of the last half of the last century and which still, essentially, forms the foundation of the current taxonomy of these organisms.

The next milestone in the development of *Bacillus* systematics was the publication of the *Approved List of Bacterial Names* (Skerman *et al.* 1980). In this, the number of species recognized increased to 31. Six years later the number in *Bergey's Manual of Systematic Bacteriology* had climbed further to 40 validated species, with another 27 *incertae sedis* (Claus & Berkeley 1986). This was still a relatively small number compared to that in the 5th edition of *Bergey's Manual*, but this was not a reflection of a satisfactory taxonomic arrangement.

One of the major problems with the genus *Bacillus* was that it was clearly heterogeneous. As noted in the 8th edition of the *Manual* (Gibson & Gordon 1974), it embraced, as compared with other genera, organisms with a great diversity of properties. Confirming its lack of homogeneity was a range of DNA base composition of over 30% (Claus & Berkeley 1986), as opposed to the agreed upper limit for a homogenous genus of 10% (Bull *et al.* 1992) and there were numerical studies such as that by Logan and Berkeley (1984) suggesting that the genus should be separated into five or six genera. Not inconsistent with all this were a number of proposals made between 1889 and 1952 for at least five new genera that included species usually regarded as belonging to *Bacillus* (see Gibson & Gordon 1974); none of these, however, became established.

Table 1.2 Aerobic endospore-forming genera included in the 8th edition of *Bergey's Manual*, and closely related to *Bacillus* but morphologically or physiologically different from it.

Sporolactobacillus	Kitahara & Suzuki (1963)
Sporosarcina	Kluuyver & van Niel (1936)
Thermoactinomyces	Tsiklinsky (1899)

Table 1.3 Trichome-forming bacteria from the guts of animals, and said to form endospores.

'*Anisomitus*'	Grassé (1925)
'*Arthromitis*'	Leidy (1850)
(='*Entomitus*')	Grassé (1924)
'*Bacillospira*'	Hollande (1933)
(='*Sporospirillum*')	Delaporte (1964)
'*Coleomitus*'	Duboscq & Grassé (1930)
'*Metabacterium*'	Chatton & Pérard (1913)

Another problem area concerned related genera. There were three genera of aerobic endospore-formers, *Sporolactobacillus*, *Sporosarcina* and *Thermoactinomyces* (table 1.2), which, although morphologically or physiologically very different from *Bacillus*, had been established by molecular studies to be close relatives of species belonging to this genus (Herndon & Bott 1969; Pechman *et al*. 1976; Fox *et al*. 1977; Stackebrandt & Woese 1981; Stackebrandt *et al*. 1987).

Pasteuria too was described as endospore-forming although it was very different from *Bacillus*. The taxonomy of *Pasteuria* was confused (see Sayr & Starr 1989), but some molecular evidence indicated that *Pasteuria penetrans* is a deeply rooted member of the *Bacillus*/*Clostridium* line of descent. It is, however, not related closely either to the true endospore-formers or to the actinomycetes (E. Stackebrandt, pers. comm.).

In addition, there were reports of several trichome-forming bacteria, said to form endospores, isolated from the gut of animals. These organisms have not been obtained in pure culture and their oxygen relationships have not been established (table 1.3). The spores of one of these, *Metabacterium polyspora*, show some cytological similarities to the endospores of *Bacillus* (Robinow 1951).

Finally, affinity between one *Bacillus* species and nonspore-forming organisms such as *Caryophanon latum*, *Filibacter limicola* and *Planococcus citreus* was demonstrated by Stackebrandt and his colleagues (1987).

In short, *Bacillus* was part of a very large and very diverse group of organisms and by 1986 Claus and Berkeley, recognizing the almost irresistible temptation to publish proposals to split the genus on the basis of the existing evidence, suggested that, as some areas of the genus were as yet inadequately studied, premature division of the genus should be avoided as it could cause difficulties for practitioners working with *Bacillus*.

Table 1.4 Recently described genera which include species once assigned to the genus *Bacillus*.

Alicyclobacillus	Wisotzkey *et al.* (1992)
Aneurinibacillus	Shida *et al.* (1996)
Brevibacillus	Shida *et al.* (1996)
Gracilibacillus	Wainö *et al.* (1999)
Paenibacillus	Ash *et al.* (1994)
Salibacillus	Wainö *et al.* (1999)
Virgibacillus	Heyndrickx *et al.* (1998)

Table 1.5 Genera containing endospore-forming species not transferred from *Bacillus*.

Ammoniphilus	Zaitsev *et al.* (1998)
Amphibacillus	Niimura *et al.* (1990)
Halobacillus	Spring *et al.* (1996)
Sulfobacillus	Golovacheva & Karavaiko (1991)
Thermobacillus	Touzel *et al.* (2000)

Recent changes

Evidence relating to the phylogeny of *Bacillus* has been accumulating since the early days of the application of molecular techniques to bacterial systematics, and division of the genus might have started much earlier than it did. Any such attempt, however, would probably have been unsatisfactory as until 1991 there was 16S rRNA oligonucleotide cataloguing information for only nine *Bacillus* species. Whether or not the plea for restraint in relation to division of the genus (Claus & Berkeley 1986) had any influence or not, no attempt at division occurred until 1991. In that year, Ash and her colleagues published results of studies on rRNA sequences of single strains of 51 *Bacillus* species. These showed the existence of at least five phylogenetically distinct clusters which, these authors suggested, would provide the basis for the division of *Bacillus* into several phylogenetically distinct genera.

In the next eight years, seven new genera were established, some of them based on these clusters of Ash and her co-workers (table 1.4). Also, both before and during this period, five other new genera were described for aerobic endospore-forming species not previously classified as members of the genus *Bacillus* (table 1.5). Added to *Bacillus* itself, this gives a total of 13 genera containing organisms that would probably once have been included in this genus. Leaving aside both the genera based on organisms which apparently produce endospores but whose relationships with *Bacillus* are currently completely unknown, and the nonendospore-forming species *Caryophanon latum*, *Filibacter limicola* and *Planococcus citreus*, but adding *Sporolactobacillus*, *Sporosarcina* and *Thermoactinomyces*, this brings the number of genera to 16.

Regrettably, not all of these genera meet the standards suggested by Ruth Gordon; indeed, some of them (see Chapters 8 and 9) are based on single strain species and others on so few strains that there is no possibility of assessing the

limits of variability of the taxa. This points to the need to try to isolate, from a variety of environments, additional representatives of the taxa that are poorly represented in culture collections. In doing this, though, it is hoped that such bacteriology as practiced by Leidy (1850) can be avoided. When studying '*Arthromitis*,' he wrote: 'Whilst the legs of fragments of the animals were yet moving upon my table, or one half the body even walking, I have frequently been examining the plants growing upon the intestinal canal of the same individual'!

Applied aspects

Challenging though their work is in itself, systematists must not lose sight of the needs of practitioners in applied areas, for whom classification and nomenclature are both means to an end, and not ends in themselves.

The chapters which follow contain accounts by practitioners or practitioner/systematists working with, on the one hand, some of the more important beneficial uses of *Bacillus* species as sources of insecticides (Chapters 11 and 13) or genes used to produce insect resistant plants (Chapter 12), as sources of enzymes for a variety of uses (Chapter 14) and as growth promoters for plants (Chapter 15), and on the other, some of the main but less desirable activities of aerobic endospore-formers, as causes of disease in humans (Chapter 4) and in causing problems in the dairy and food industries (Chapter 6).

The future

Division of the genus *Bacillus sensu lato* is now so substantial that in a sense *Bacillus* has withered! The number of genera derived from it, however, give perhaps a misleading impression of a reduction in its size. In fact, there are actually still more species in *Bacillus* than in all the genera containing close relatives, some transferred from *Bacillus* and some newly described, put together.

As suggested in Chapter 2, partition of some clearly heterogeneous species is likely to be a next area of activity.

Further ahead, one (probably safe) prediction is that, given the number of taxa with very small numbers of representative strains or species, there will be retrenchment, and perhaps publication of a new version of the *Approved List of Bacterial Names*. This would follow logically from a consensus being arrived at concerning the concept of the bacterial species.

It is equally certain that this process of cutting back the number of taxa will be informed by phylogenetic information and that the species of aerobic endospore-forming bacteria, let alone their close relatives, will probably never, as hoped by Ruth Gordon (1981), number fewer than the 146 listed in the fifth edition of *Bergey's Manual*.

As is implied in, for example, Chapters 8 and 17, it is desirable, if this is indeed possible (see Chapter 19), that there be agreement about the concept of the bacterial species, reconciling that based on molecular approaches to systematics with those depending on other approaches. There will be a need to address questions

such as: 'Should taxa which are based on phylogenetic information, and which cannot be identified by any known phenotypic tests, be validly described?' and 'Is there a desirable minimum number of strains on which to base valid species?'. The discussion will be fascinating and important if the tensions (see Chapter 2) caused by the two approaches are to be reduced or eliminated.

Whatever the outcome of work and debates to that end, I am sure, given the desirability of the predictive value of nomenclature, and, not overlooking the existence of genera such as *Lactobacillus, Streptobacillus, Thiobacillus*, etc., that generic epithets for aerobic endosporers should contain the root *-bacillus* (cf. *Ammoniphilus*) continuing its association with this important group of bacteria.

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Chapter 2

From Phylogeny to Systematics: the dissection of the genus *Bacillus*

Erko Stackebrandt and Jolantha Swiderski

Introduction

Unexpected relationships between members of *Bacillus* and other genera such as *Sporosarcina* were first revealed by Fox *et al.* (1977) on the basis of comparative 16S rDNA cataloguing. Over the following decade the main outlines of prokaryotic phylogeny became available, in which *Bacillus* species were shown to cluster with other taxa of Gram-positive bacteria which exhibit a low DNA base composition (<50 mol% G+C) (Ludwig & Schleifer 1994; Olsen *et al.* 1994). Despite this tremendous progress in our understanding of bacterial phylogeny, the interpretation of molecular data is not straightforward, depending as it does on mathematical algorithms, selection and number of reference sequences and selection of sequence positions. For example, the phylogenetic coherency of the Gram-positive bacteria – the so-called *Clostridium/Bacillus* subline and the Actinobacteria subline – have still to be shown convincingly (Van de Peer *et al.* 1994). Studies on genes and gene products other than ribosomal RNA genes have been performed mainly on *B. subtilis*. Analyses of genomic properties do not, therefore, contribute significantly to the phylogeny of the genus *Bacillus*, but this will most likely change as more species are included in genome sequencing projects. With one exception, *B. subtilis* does indeed group with Gram-positive reference organisms which exhibit low G+C contents; e.g. in studies on RNases H (Ohtani *et al.* 1999), family C DNA polymerases (Huang & Ito 1998), DNAK (heat shock protein) (Gupta *et al.* 1997), GroEL (chaperonin) (Viale *et al.* 1994; Dale *et al.* 1998) and σ^{70}-type sigma factors (Gruber & Bryant 1997). In contrast, analysis of *nifH* genes (Achouak *et al.* 1999) showed members of *Paenibacillus* to cluster next to cyanobacteria, while members of *Clostridium*, their relatives according to 16S rDNA and other genes and proteins (Olsen *et al.* 1994; Van de Peer *et al.* 1994), grouped only distantly.

The order in which branches diverge from each other is a matter for discussion, ranging from the most remotely related lineages to the fine details of taxa that have evolved recently. While the influences of certain factors on tree topologies are known to experienced taxonomists, the neophyte is often puzzled by changes in the positions of taxa within phylogenetic dendrograms. This chapter shows the effect of commonly used algorithms on tree topologies within the *Bacillus* cluster, and tries to explain that despite certain uncertainties in the position of most deeper-branching lineages, recent changes in the systematics of these organisms are, by and large, justified from a phylogenetic point of view.

Reclassification based upon phylogenetic diversity

Despite major revisions in the taxonomy of *Bacillus*, the taxonomic entity of this genus as defined at the time of its original description (Cohn 1872) still exists in the description of the type species *B. subtilis* and phylogenetically affiliated species. Actually, the vast majority (88%) of the 114 species described as members of this genus up to the year 2000 are still members of the genus *Bacillus*. Following the pioneering study of Ash *et al.* (1991) – which itself was a continuation of earlier studies by Fox *et al.* (1977), Clausen *et al.* (1985), Stackebrandt *et al.* (1987) and others – some of the phyogenetically distinct entities were later reclassified as new genera (present number of species in brackets), i.e. *Alicyclobacillus* (3), *Aneurinibacillus* (3), *Brevibacillus* (10), *Gracilibacillus* (2), *Halobacillus* (3), *Paenibacillus* (24), *Salibacillus* (1) and *Virgibacillus* (2). Other organisms, which formerly would have been placed in *Bacillus,* were described as members of novel genera, such as *Thermobacillus* (1) and *Amphibacillus* (1). The dissection of *Bacillus* followed a trend that brought taxonomy on a par with phylogeny. As a consequence, traditional key characters, such as rod-shaped morphology, aerobic metabolism and spore formation lost their significance in circumscribing the genus. Some taxonomists may disagree with this change in dealing with taxonomy, and a look through the microscope and determination of growth properties may still be faster than determination of the primary structure of 16S rDNA and subsequent phylogenetic analyses. However, today, the main concern is directed less towards the dissection of the genus and more towards the splitting of strain-rich species, and species clusters, in which either DNA–DNA reassociation similarities or the presence of subspecific, genomically coherent traits guide the splitting process. These decisions are often not accompanied by the description of sufficient phenotypic properties for a diagnostic laboratory to identify a strain or recognize it as a representative of a novel species. It is in this field of tension, also seen with other groups of organisms, that microbiologists are currently asked to do taxonomic work: on the one hand, using the potential of doing detailed molecular analyses, down to the level of strains, even clones; and on the other, knowing that the tools for unravelling these genomic properties will for a long time be unavailable to the majority of users worldwide.

Assessing the taxonomic boundaries of the genus *Bacillus* and related taxa

The vast majority of 16S rDNA dendrograms including members of *Bacillus* have been based upon distance analyses, using the Jukes and Cantor (1969) correction of similarity values. It is not surprising, therefore, that the topologies of the phylogenetic patterns, generated by basically the same method, differ from each other only in detail in different publications. *Bacillus* species were found to form clusters that have been named RNA groups 1 to 5 (Ash *et al.* 1991). Later, the presence of an additional RNA group – named group 6 – has been described for alkaliphilic and alkalitolerant species (Nielsen *et al.* 1994). Some of these groups,

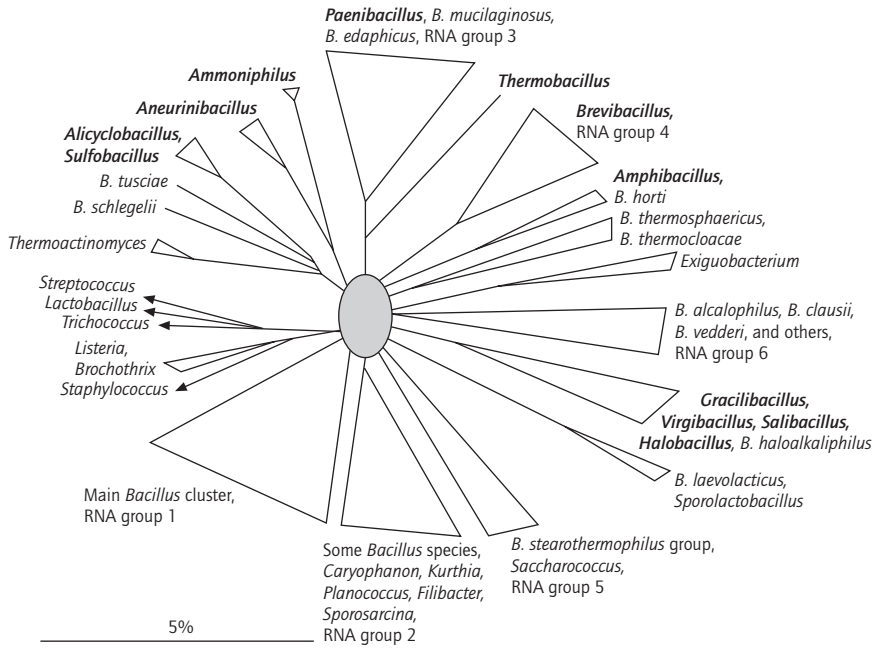

Figure 2.1 Schematic outline of the phylogenetic diversity of 16S rDNA of aerobic, rod-shaped and spore-forming, Gram-positive bacteria, classified as species of *Bacillus*, genera that originated from the dissection of *Bacillus,* and species that were affiliated to novel genera because of their distinct phylogenetic positions. The areas of the triangles represent approximations of the number of species included in the taxa covered by the triangle. The circle indicates the uncertainty of the order at which the lineages diverge from each other. *B*, *Bacillus*.

such as *Paenibacillus* (group 3), and *Brevibacillus* (group 4), have been reclassified since 1991, while other separate lineages have been reclassified as *Aneurinibacillus, Alicyclobacillus, Halobacillus, Gracilibacillus, Salibacillus* and *Virgibacillus*. The main radiation of these organisms, based upon neighbour-joining analysis (Felsenstein 1993) is shown schematically in figure 2.1. This dendrogram also depicts the presence of non-*Bacillus* genera among *Bacillus* groups and clusters, and identifies potential new genera for those species which are not related to those already reclassified, e.g. *B. tusciae, B. schlegelii, B. horti, B. laevolacticus, B. thermocloacae*, and members of RNA group 6.

Although the number of sequences of type strains has been increased significantly during recent years (from about 50 by Ash *et al.* 1991 to >120 in 2000), the groups defined in 1991 still, by and large, emerge in any of the phylogenetic analyses published. However, as the topology of a dendrogram depends strongly upon the overall number of sequences and the number of sequences in any particular group, the order in which they emerge in the dendrogram may differ significantly between studies. Rather than showing the precise branching order at deeper phylogenetic levels as unravelled by the phylogenetic analysis, this region is not further resolved in figure 2.1. Figures 2.5 and 2.6 are more detailed phylogenetic analyses based upon the neighbour-joining method (see below).

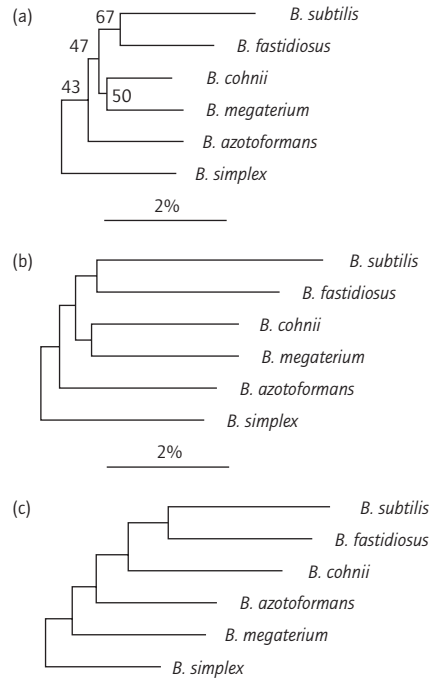

Figure 2.2 Comparative analysis of 16S rDNA of six type strains of *Bacillus* species of RNA group 1. Bar indicates 2% nucleotide substitutions. (a) Distance-matrix analysis using the least squares algorithm of DeSoete (1983) and the Jukes and Cantor (1969) correction to compensate for different evolutionary rates. The four highest bootstrap values are indicated. (b) Distance-matrix analysis using the neighbour-joining method (Felsenstein 1993) and the Jukes and Cantor (1969) correction to compensate for different evolutionary rates. (c) Maximum-likelihood analysis, using the program DNAML (transition–transversion rate 2.000) (Felsenstein 1993). *B*, *Bacillus*.

Different treeing algorithms generate different topologies

Algorithms such as DNAMI, included in PHYLIP (Felsenstein 1993), have been used only rarely to determine the phylogenetic relatedness of *Bacillus* species and related taxa, probably owing to the long computing time required to analyse dozens of sequences by the maximum-likelihood method. Distance-matrix programs such as NEIGHBOR use dissimilarity values to correct for rate variation, while maximum-likelihood methods estimate phylogenies from nucleotide sequences. The latter model allows for unequal expected frequencies of the four nucleotides and for different rates of change in different categories of sites. Figure 2.2 compares the branching patterns of a small set of species from RNA group 1, which present phylogenetically well-separated taxa (93.8–96.3% 16S rDNA sequence similarity). Figures 2.2a and 2.2b have been generated by distance-matrix analyses using the Jukes and Cantor (1969) correction to compensate for different evolutionary rates; figure 2.2a is based on the algorithm of DeSoete (1983), while figure 2.2b is a neighbour-joining (NJ) dendrogram (Felsenstein 1993). The bootstrap values presented in figure 2.2a are low, indicating a low degree of statistical significance in the branching order. Figure 2.2c is a maximum-likelihood (ML) dendrogram (Felsenstein 1993). Quite obviously, the two distance-matrix trees have similar topologies, while the ML dendrogram differs in the branching of *B. megaterium* and *B. azotoformans*.

One can assume that differences in the topologies of dendrograms are further increased when less-related species are included in the analyses. Figure 2.3

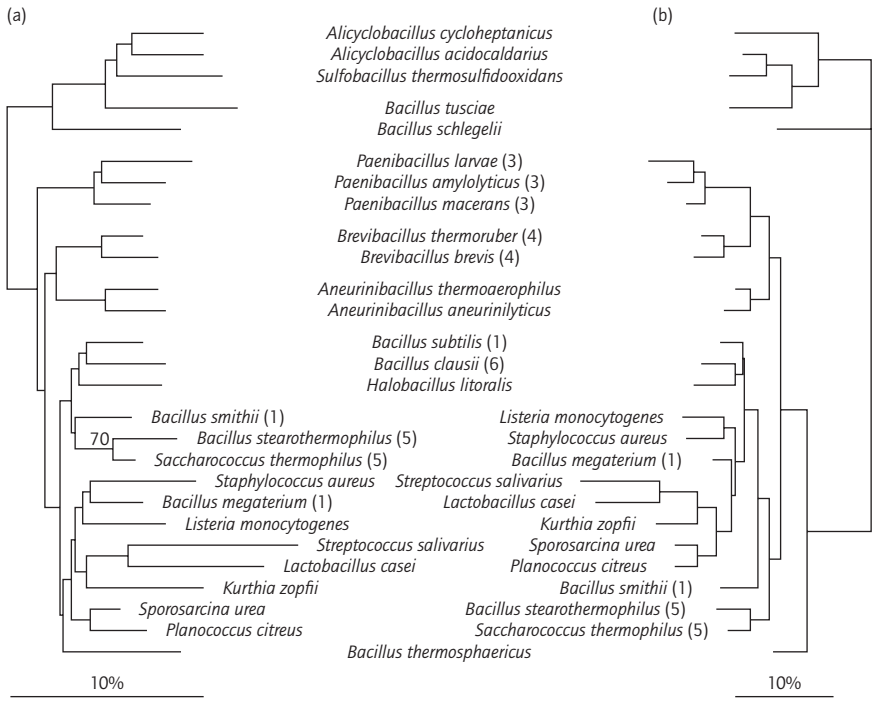

Figure 2.3 Comparative analysis of 16S rDNA of a broad selection of type strains of *Bacillus* species and non-*Bacillus* reference strains. The sequence of *Paenibacillus amylolyticus* has been generated from the nontype strain NCIMB 8144. The bar indicates 10% nucleotide substitutions. (a) Distance-matrix analysis using the neighbour-joining method (Felsenstein 1993) and the Jukes and Cantor (1969) correction to compensate for different evolutionary rates. (b) Maximum-likelihood analysis, using the program DNAML (transition–transversion rate 2.000) (Felsenstein 1993).

compares the topologies of dendrograms of 27 species of *Bacillus* and reference taxa, generated by the NJ dendrogram with the Jukes and Cantor (1969) correction (figure 2.3a) and the ML method (transition–transversion rate 2.000) (figure 2.3b). Both dendrograms are similar in some details but differ significantly in others: most of the closely related species group together in both dendrograms, e.g. *Paenibacillus*, *Brevibacillus*, *Aneurinibacillus, B. stearothermophilus* and *Saccharococcus thermophilus*, while significant differences occur at deeper levels of relationship. This is not only demonstrated by the intergeneric relationships of *Paenibacillus, Brevibacillus, Aneurinibacillus* and most of the non-*Bacillus* reference taxa, but also by members of RNA group 1, which do not appear to form a phylogenetically coherent cluster. Bootstrap values calculated for branching points of the NJ dendrogram are low in most cases, indicating the low statistical significance of the order at which they separate. It should be noted that high bootstrap values are no proof *per se* of exclusive phylogenetic relatedness; they demonstrate that the same branching order is recovered in most of the sub-trees recovered in the analysis. High bootstrap values (>90%) are likely to occur in

those cases where lineages are separated from each other by long internodes from neighbouring lineages. The addition of new sequences would most likely change this apparent proof of phylogenetic evidence. For this reason bootstrap values are not indicated in figures 2.5 and 2.6.

Another method of displaying the statistical significance of phylogenies makes use of multiple datasets, generated by bootstrap resembling, which themselves serve as input files for a program that estimates phylogenies by the parsimony method. Figure 2.4 is a consensus tree based upon analyses of 100 bootstrapped datasets included in the DNAPARS program. When compared to the topologies of the NJ and ML dendrograms (figure 2.3), certain topological features are reproduced, such as the separate clustering of *Sulfobacillus, Alicyclobacillus, B. tusciae* and *B. schlegelii*. Members of *Paenibacillus*, *Brevibacillus* and *Aneurinibacillus* also form recognizable entities, while the branching order of the taxa included in the boxed area differ in all three dendrograms. Considering that only a single representative has been selected from each of the species-rich genera *Lactobacillus, Streptococcus* and *Staphylococcus,* additional changes might be expected to occur following their inclusion in the analyses.

As a consequence, phylogenetic patterns derived by any of the several methods available today give no 'proof' that the topologies closely reflect the course of evolution. The closer the matches in the topologies of dendrograms generated by different algorithms, such as distance-matrix analyses, maximum-likelihood and parsimony, the higher the chance that the branching patterns do indeed express phylogenetic evidence. This is clearly the case for the emergence of individual genera described during the past years, as well as for several individual lineages that will probably be described as novel genera in the future, e.g. *B. schlegelii* and *B. tusciae*. The new generation of personal computers will handle even the time-costly maximum-parsimony algorithms better, and future conclusions about taxonomic relatedness among species should be based on more than just a single tree-inferring approach.

Phylogenetic grouping and phenotypic circumscription

The phylogeny-based dissection of *Bacillus* RNA groups is in many cases not supported by clear-cut phenotypic properties. This finding may be of concern to some taxonomists, as affiliation of novel strains to the respective taxa is dependent mainly upon phylogenetic analysis of 16S rDNA (similarity values and signature nucleotides) and the occurrence of PCR fragments in gel electrophoresis following PCR amplification using genus-specific primers (Shida *et al.* 1996). In only a few cases are salient characteristics such as distinct chemotaxonomic properties available for genus affiliation, examples being the amino-acid composition of peptidoglycan in members of *Halobacillus* (Spring *et al.* 1996), or ω-alicyclic fatty acids in *Alicyclobacillus* species (Wisotzkey *et al.* 1992). In the case of *Thermobacillus* (Touzel *et al.* 2000), a sister group of the *Paenibacillus* lineage, the genus description is so poor in descriptive features that its members cannot be affiliated to the genus without the help of 16S rDNA data. Members of yet other genera, such as *Amphibacillus* (Niimura *et al.* 1990), *Paenibacillus* (Shida *et al.*

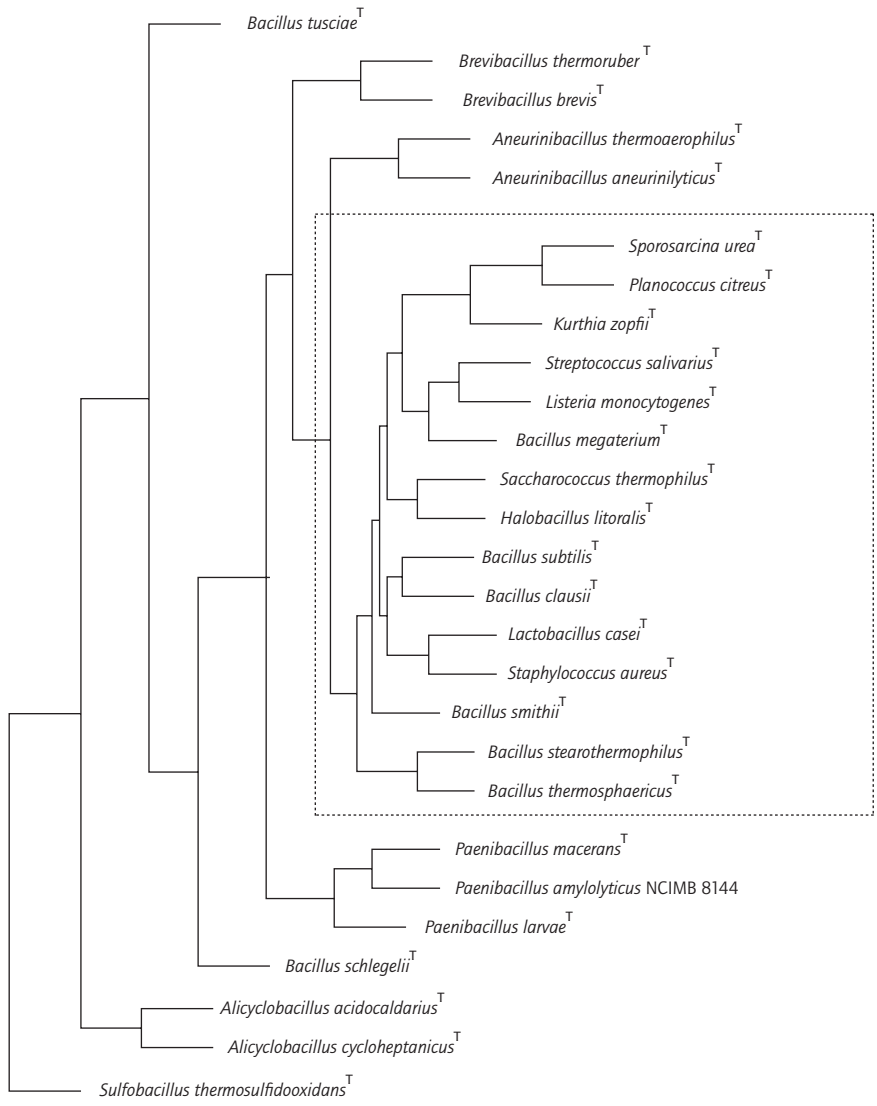

Figure 2.4 Consensus parsimony (DNAPARS; Felsenstein 1993) dendrogram of sequences included in figure 2.3, based upon 100 bootstrapped trees. The topology should be compared to those presented in figure 2.2a,b. The boxed area indicates those organisms whose branching orders are affected most obviously by the selection of treeing algorithms. T, type strain.

1997a), *Brevibacillus* (Shida *et al.* 1996), *Salibacillus* (Wainö *et al.* 1999), *Gracilibacillus* (Wainö *et al.* 1999), *Virgibacillus* (Heyndrickx *et al.* 1998) and *Aneurinibacillus* differ from each other in some phenotypic characteristics, but these are not exclusive in most cases. Moreover, a comparative listing of genus-specific properties (Heyndrickx *et al.* 1998) indicates that several of these properties have not yet even been elucidated for members of all genera. In fact, the

phenotypic properties listed as genus-specific are usually those used to describe species in other genera of Gram-positive bacteria.

Bacillus RNA group 1

This group constitutes the core of *Bacillus*, containing the name-bearing type species *B. subtilis* (figure 2.5). As already indicated by Ash *et al.* (1991), the phylogenetic diversity of this group is huge, encompassing several well-separated species clusters and single-species lineages. When selected single species are included in a larger database of reference organisms, they do not necessarily cluster together (see figures 2.2a, 2.2b and 2.3), which may indicate that this group does not form a coherent phylogenetic entity. Were discriminating phenotypic properties to be available, RNA group 1 could be divided into several genera, as done with similarly remotely related species which were transferred to *Virgibacillus, Salibacillus, Halobacillus* and *Gracilibacillus* (figure 2.6). One of these subgroups comprises *B. vallismortis, B. mojavensis, B. subtilis, B. amyloliquefaciens, B. atrophaeus* and *B. licheniformis*; a second one contains *B. cohnii, B. horikoshii* and *B. halmapalus*; a third one harbours *B. cereus, B. pseudomycoides, B. anthracis B. thuringiensis, B. weihenstephanensis* and *B. mycoides*; while a fourth one embraces *B. simplex, B. psychrosaccharolyticus*, the invalid species '*B. maroccanus*' and two misclassified *Brevibacterium* and *Arthrobacter* species. Most of the other species of this group form more deeply rooting lineages which fan out without allowing the determination of their branching order.

Bacillus RNA group 2

This group constitutes an evolutionary enigma. *Bacillus*-type organisms are intermixed with spherical spore-formers (*Sporosarcina*) and nonspore-forming rods [*Filibacter* (Clausen *et al.* 1985; not shown in figure 2.5), *Kurthia*, *Caryophanon*] and cocci (*Planococcus*). The hallmarks of this group are the presence of either L-lysine or ornithine at position 3 of the peptide subunit and a dicarboxylic amino acid in the interpeptide bridge. The majority of the other taxa covered in this chapter (the exception being *Halobacillus*) contain a directly cross-linked peptidoglycan with *meso*-diaminopimelic acid at position 3 of the subunit. While the non-*Bacillus*-type genera within this RNA group form phylogenetically coherent entities, members of *Bacillus* cluster around *Sporosarcina* and *Caryophanon*. Considering that the ancestors of this group contained *Bacillus* RNA group 1-type characters, this finding may indicate that the *Bacillus* species of RNA group 2 have been prone to significant genomic rearrangements or other genomic changes which lead to the loss of rod-shaped morphology and spore-formation. The intermixing of phenotypically different genera with *Bacillus* species constitutes an interesting taxonomic problem. In order to make classification consistent with phylogeny, the four different lineages of *Bacillus* species (*B. globisporus* and relatives, *B. insolitus, B. fusiformis* and *B. silvestris*) would have to

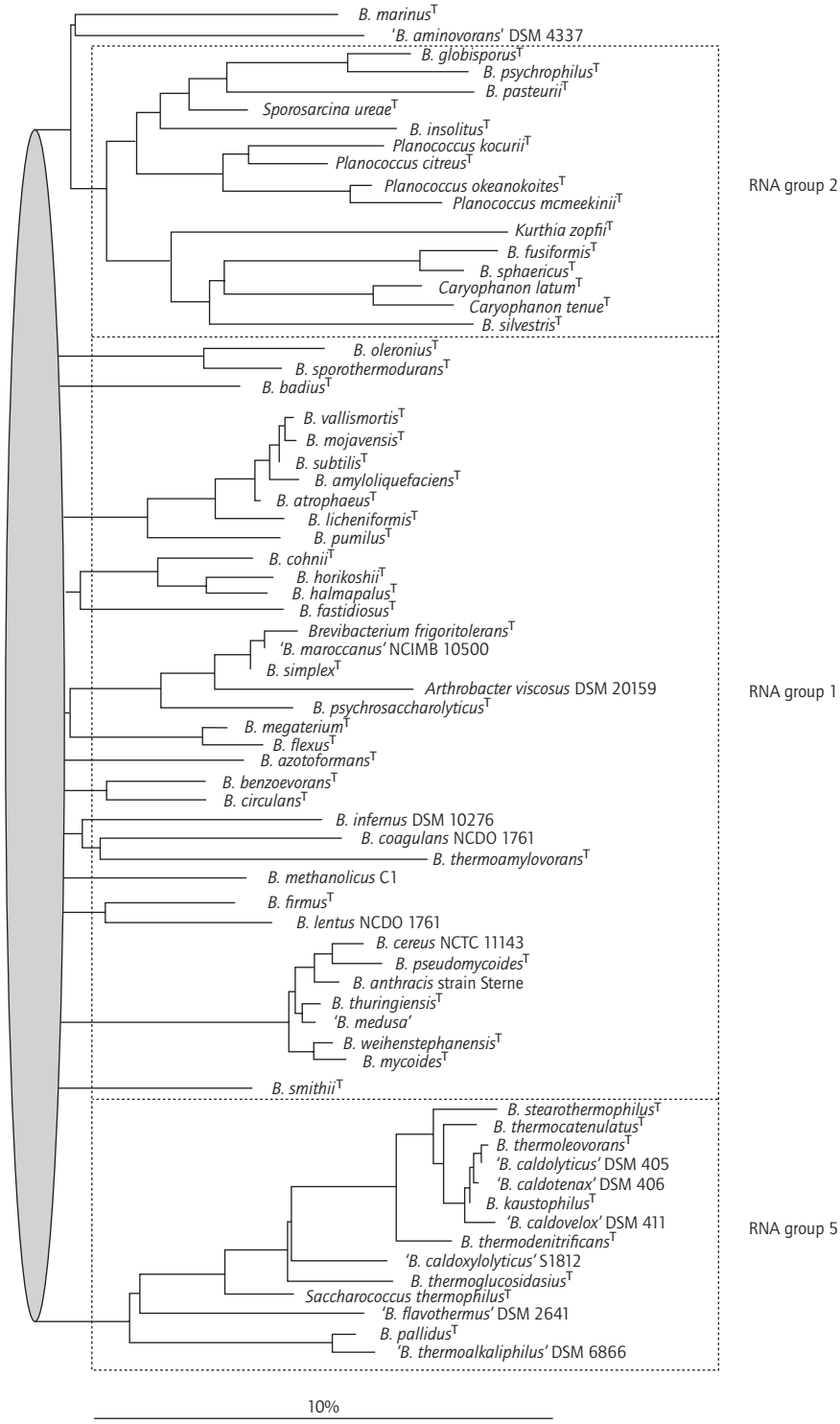

Figure 2.5 Detailed neighbour-joining tree of species of RNA groups 1, 2 and 5. The dotted area indicates the uncertainty of the order at which the lineages diverge from each other. The area was chosen somewhat arbitrarily and may just as well cover more recent branching points. The bar indicates 10% nucleotide substitutions. *B*, *Bacillus*; ^{T}, type strain.