Simplified technique for identification of the aerobic spore-forming bacteria by phenotype

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The use of modern research approaches of genetics, biochemistry and molecular biology has led to progress in bacterial taxonomy. Systematic study of the aerobic spore-forming bacteria has resulted in the realignment of the genus *Bacillus* into several new genera. In the meantime, the identification process has become more difficult for the non-specialist in *Bacillus* taxonomy. This paper presents a key for the simplified phenotypic identification of the mesophilic, aerobic, spore-forming bacteria belonging to the genera *Bacillus*, *Paenibacillus*, *Brevibacillus*, *Aneurinibacillus*, *Geobacillus* and *Virgibacillus*. A total of 81 species were included and 115 morphological and physiological tests were analysed for their discriminative efficiency. This key is practical for rough but quick identification of aerobic spore-forming bacteria isolated from nature. Such preliminary identification will be helpful for the selection of reference strains and methods for more precise identification using the newest techniques. The reliability of the proposed identification key was tested on 100 cultures from the Ukrainian Collection of Microorganisms. The developed identification key is represented in interactive mode on a website (<http://www.imv.kiev.ua/key/>).

Keywords: identification, phenotype, spore-forming bacteria

INTRODUCTION

Aerobic spore-forming bacteria represent a major microflora in many natural biotopes, where they play an important role in ecosystem development; they are able to transform many chemical compounds, including industrial pollutants. Therefore, the investigation of these micro-organisms is highly important for microbiologists dealing with ecological studies and environment protection. The identification of aerobic spore-forming bacteria was always a difficult task, but has recently become almost impossible to the non-specialist in the field of bacillus systematics and identification. Many of the up-to-date, sophisticated genetic and biochemical methods that have permitted improvements in bacilli taxonomy are too difficult for routine use. A prominent trend of current microbiological practice is the identification of micro-organisms based only on 16S rRNA sequences, which provides rather limited resolution (Schloter *et al.*, 2000; Stackebrandt & Goebel, 1994). Uncritical use of results of 16S rRNA gene comparison can lead to

incorrect identification and, more dangerously, to incorrect association of DNA sequences in a genetic database with particular species, which will multiply the error many times. It would be useful to supply scientists with a method to check quickly whether their strains identified by 16S rRNA agree with the corresponding species characteristics.

In recent years, twelve new genera of aerobic spore-forming bacteria have been proposed: *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Amphibacillus* (Niimura *et al.*, 1990), *Aneurinibacillus* and *Brevibacillus* (Shida *et al.*, 1996), *Filobacillus* (Schlesner *et al.*, 2001), *Geobacillus* (Nazina *et al.*, 2001), *Halobacillus* (Spring *et al.*, 1996), *Paenibacillus* (Ash *et al.*, 1993), *Virgibacillus* (Heyndrickx *et al.*, 1998), *Gracilibacillus* and *Salibacillus* (Wainø *et al.*, 1999) and *Ureibacillus* (Fortina *et al.*, 2001). Many species and genus names used routinely in the current scientific literature are not present in *Bergey's Manual of Systematic Bacteriology* (Claus & Berkeley, 1986). This situation causes difficulties in identification of any bacillus. In addition, some authors have described new species based on significantly different sets of physiological and morphological characters, adding to the problem of

Abbreviation: VP, Voges–Proskauer.

Table 1. Strains used in this study

Strains were obtained from the following culture collections: ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; UCM, Ukrainian Collection of Microorganisms, Kyiv, Ukraine; VKM, All-Russian Collection of Microorganisms, Pushchino, Russia. Strain accession numbers are from the UCM or are our work numbers unless otherwise stated.

Species	Strains
<i>Bacillus badius</i>	UCM B-5820
<i>Bacillus cereus</i>	UCM B-5650 ^T (DSM 31 ^T); 5652; 5653; 5655; 5656; 5659; 5671; 5673
<i>Bacillus cereus</i> var. <i>mycoides</i>	UCM B-5674
<i>Bacillus circulans</i>	UCM B-5841 (ATCC 9966); 5845 (VKM B-729)
<i>Bacillus coagulans</i>	UCM B-5850 ^T (DSM 1 ^T)
' <i>Bacillus endophyticus</i> '	UCM B-5715; 5908; 5910; 5912
' <i>Bacillus entomocidus</i> '	UCM B-5900 (ATCC 19268)
<i>Bacillus lentus</i>	UCM B-5906 ^T (DSM 9 ^T)
<i>Bacillus licheniformis</i>	UCM B-5501 ^T (DSM 13 ^T); 5502; 5503; 5506; 5508; 5510; 5511; 5512; 5513; 5514
' <i>Bacillus longissimus</i> '	UCM B-5880 (DSM 352)
<i>Bacillus megaterium</i>	UCM B-5723 (ATCC 9885); 5714; 5716; 5718; 5719
<i>Bacillus pumilus</i>	UCM B-5604 ^T (DSM 27 ^T); 5601; 5602; 5609; 5610; 5617
<i>Bacillus sphaericus</i>	UCM B-5901 ^T (DSM 28 ^T); 5902
<i>Bacillus subtilis</i>	UCM B-5006 ^T (DSM 10 ^T); 5001 (ATCC 10774); 5002; 5007; 5008; 5009; 5010; 5012; 5014; 5016; 5017; 5018; 5019; 5021; 5029; 5036; 5042; 5044; 5050; 5071; 5074; 5075; 5121; 5111; 5112; 5113; 5127
<i>Bacillus thuringiensis</i> var. <i>sotto</i>	UCM B-5680 ^T (ATCC 19270 ^T)
<i>Bacillus</i> sp.	UCM B-5530; 5843; 5844; 5715; 5908; 5910; 5911; 9DT; 7-91D; 76D; 103D; 106D; 107D; 108D; 109D; 111D; 115D; 119D; 121D; 1r; 2r
<i>Brevibacillus brevis</i>	UCM B-5830 ^T (DSM 30 ^T)
<i>Brevibacillus laterosporus</i>	UCM B-5870 ^T (DSM 25 ^T)
<i>Paenibacillus alvei</i>	UCM B-5904
<i>Paenibacillus macerans</i>	UCM B-5803 ^T (DSM 24 ^T); 5800
<i>Paenibacillus polymyxa</i>	UCM B-5760 ^T (DSM 36 ^T); 5756; 5757; 5758; 5759
<i>Paenibacillus pulvifaciens</i>	UCM B-5909 (VKM B-748)
<i>Virgibacillus pantothenicus</i>	UCM B-5907 (DSM 26)

comparing phenotypes. It therefore seemed useful to review and summarize the current taxonomic and identification schemes developed for aerobic spore-forming bacteria. In this review, both approved and newly proposed species names are included. It is, however, beyond the scope of this paper to discuss the worth of the use of any of these taxonomic names. The review is intended to discuss some aspects of the phenotypic heterogeneity of the bacilli and to analyse the suitability of several phenotypic tests for identification of aerobic spore-forming bacteria. Our purpose was to put together the scattered descriptions of all known species of *Bacillus* and related genera in order to provide a method by which an idea of what an unknown strain could be can be formulated, before using more complicated and expensive methods of investigation. We want to underline that the key proposed below is targeted to the rough identification of aerobic spore-forming micro-organisms, and further detailed identification may be required.

The paper deals with mesophilic spore-forming bacteria growing on nutrient agar at neutral pH under aerobic conditions at temperatures of 25–45 °C. We

have therefore not included in this discussion representatives of such genera as *Alicyclobacillus* (thermophiles and acidophiles), *Amphibacillus* and *Ureibacillus* (alkaliphiles), *Halobacillus*, *Filobacillus*, *Gracilibacillus* and *Salibacillus* (halotolerant bacteria) and several extremophilic, asporogenous and nutritionally fastidious *Bacillus*, *Geobacillus* and *Paenibacillus* species.

METHODS

Selection of phenotypic tests for the identification key.

Information obtained from the literature concerning 81 species of aerobic spore-forming bacteria characterized by 115 physiological and morphological tests was accumulated and summarized in a database.

A criterion to determine the discriminative efficiency of a test was calculated from the following equation:

$$E_i = 4 \times \left(\bar{q}_i(1 - \bar{q}_i) - \frac{\sum q_{it}(1 - q_{it})}{N} \right)$$

where $\bar{q}_i$ is the ratio of positive responses of test i for all species; q_{it} is the ratio of positive responses for test i for strains of species t ; and N is the total number of species in the

Table 2. Tests ranked by their discriminative efficiency for the identification of aerobic spore-forming bacteria

E represents the efficiency of a test as calculated from the equation given in Methods, whereas e represents the calculated efficiency of a test multiplied by the proportion of species that were characterized by that test.

Rank	Not taking frequency of test use into account		Taking frequency of test use into account	
	Test	E	Test	e
1	Resistance to nalidixic acid (32 µg ml ⁻¹)	0.93	Degradation of starch	0.86
2	Resistance to polymyxin (16 µg ml ⁻¹)	0.91	Growth at 50 °C	0.80
3	Anaerobic growth	0.89	Nitrate reduction	0.75
4	Degradation of starch	0.89	Sporangium bulging	0.75
5	Degradation of Tween 20	0.88	Anaerobic growth	0.73
6	Growth at 50 °C	0.87	Degradation of casein	0.72
7	Degradation of casein	0.86	Growth with 5% (w/v) NaCl	0.66
8	Acid from L-arabinose	0.85	Gelatin liquefaction	0.65
9	Growth with 5% (w/v) NaCl	0.85	Acid from L-arabinose	0.61
10	Oxidase activity	0.85	Length of cell > 3 µm	0.61
11	Acid from melibiose	0.84	Growth at pH 6.0	0.59
12	Acid from raffinose	0.84	Acid from xylose	0.59
13	pH Voges-Proskauer broth < 7	0.83	Utilization of citrate	0.58
14	Utilization of succinate	0.83	Oxidase activity	0.58
15	Acid from mannitol	0.83	Voges-Proskauer reaction	0.55
16	Growth at pH 6.0	0.82	Acid from raffinose	0.53
17	Nitrate reduction	0.82	Growth at 28 °C	0.52
18	Acid from xylose	0.82	Acid from salicin	0.52
19	Acid from salicin	0.81	Acid from galactose	0.51
20	Degradation of Tween 80	0.81	Acid from lactose	0.48

database. Of all possible tests, the most efficient would be a test for which the value of E_i is 1, in the case where a given number of species is divided into two equal groups (positive and negative by this test) with no variable responses. If all of the species are positive or negative by this test or if the variability of the test between several species is the same as that within this species, the value of E_i is zero.

Bacterial strains. The identification test that we developed was tested on strains isolated from different ecological niches and contained in the Ukrainian Collection of Micro-organisms (UCM). These strains are represented in Table 1 by their catalogue number (if available) or by work numbers for fresh isolates.

Phenotypic characterization. Strains were tested as described by Claus & Berkeley (1986) and Gordon *et al.* (1973) by using the following characteristics: cellular and spore morphology; catalase and oxidase activity; Voges-Proskauer (VP) test; nitrate reduction to nitrite; anaerobic growth; gas production from nitrate and glucose; starch, urea and casein degradation; gelatin liquefaction; acid production from glucose, galactose, lactose, mannose, mannitol, salicin and fructose; citrate utilization; decomposition of tyrosine; arginine dihydrolase activity; growth with 5% (w/v) NaCl and with 0.001% (w/v) lysozyme.

Genotyping. Ribotyping was performed as described by Aquino de Muro & Priest (1993). Arbitrary primer PCR (RAPD typing) was carried out as described by Brousseau *et al.* (1993). Primer 0955-03 (5'-CCGGCGGCG-3') was used for amplification.

RESULTS AND DISCUSSION

In Table 2, the phenotypic tests available are shown in order of decreasing discriminative efficiency (E). On the right of the table, the same tests are ranked by the discriminative efficiency multiplied by n_i/N (e), where n_i is the number of species characterized by test i and N is the total number of species in the database. Only the 20 most efficient phenotypic tests from 115 analysed are represented in Table 2. It is therefore clear from this table that the bacilli were divided most efficiently by such tests as resistance to nalidixic acid (32 µg ml⁻¹) and to polymyxin (16 µg ml⁻¹). However, only 34 species of bacillus were characterized by these tests (Priest *et al.*, 1988), so they are not included in the list of the 20 most efficient tests as ranked by e (Table 2).

The selection of tests for the identification key was based on their discriminative efficiency and their usual application in routine laboratory characterization of aerobic spore-forming micro-organisms. Having set the tests for one level of the key, the discriminative efficiencies of the remaining tests were recalculated for each group obtained. While selecting tests, the correlative independence of any pair of tests was also taken into account. Another goal was to minimize the number of key tests and to choose, if possible, tests that occurred elsewhere in the key, even if they were

Anaerobic growth (-)

slightly less effective. Furthermore, phylogenetic relatedness was not considered as a basis for dividing the organisms into groups. Our only aim was to simplify species identification. The identification key is shown in Fig. 1.

Strains of some species were variable by the tests selected for the key. Such strains are represented in different cells of the scheme (Fig. 1). Thus, strains of *Bacillus cereus* are variable for starch degradation, so this species is placed in two different cells, I.A.4 and II.B.1.i. Some tests were considered as positive (negative) for a species if the proportion of strains responding positively was over 80% (did not exceed 20%).

Group I consists of facultatively anaerobic micro-organisms incapable of starch hydrolysis (Fig. 1). It is subdivided further according to the Voges–Proskauer reaction (VP). VP-positive organisms form subgroup I.A and VP-negative organisms form subgroup I.B. Subgroup I.A includes *Bacillus cereus*, *Paenibacillus azotofixans* and a group of homolactic acid-fermenting bacteria similar to ‘*Bacillus racemilacticus*’ (‘*Bacillus laevolacticus*’, ‘*Bacillus myxolacticus*’ and ‘*Bacillus dextrolacticus*’). These species were differentiated on the basis of the optical isomer of lactic acid produced; however, they were found to be strains of the same species according to data for 16S rRNA gene sequences (Suzuki & Yamasato, 1994). Nishikawa *et al.* (1996) reported that *B. cereus* strains incapable of starch hydrolysis were frequently producers of the emetic toxin, a cause of food-poisoning outbreaks.

Subgroup I.B includes insect pathogens belonging to the genera *Brevibacillus* and *Paenibacillus*, a nicotinate-metabolizing species, *Bacillus niacini*, and a nitrogen-fixing bacterium, *Paenibacillus borealis*. *Paenibacillus larvae* and *Paenibacillus pulvificiens* were reclassified as subspecies of *P. larvae* (Heyndrickx *et al.*, 1996a). Two other species belong to this subgroup, namely *Bacillus thermocatenulatus* (a thermophile that is distinguished by growth at 50 °C; Golovacheva *et al.*, 1975) and *Bacillus pasteurii* (an alkaliphile that is recognized by growth at pH 9.0 and high urease activity; Claus & Berkeley, 1986).

Group II includes facultatively anaerobic, starch-hydrolysing representatives of the genus *Paenibacillus*, thermophilic species of *Geobacillus* and the *Bacillus cereus* group, including *Bacillus thuringiensis*, *Bacillus mycoides* and *Bacillus anthracis*. Further identification was based on the following features: VP reaction, nitrate reduction, casein and gelatin hydrolysis, growth at 50 °C, oxidase and urease activities and gas production from glucose. *Virgibacillus pantothenicus* also appeared in group II; however, the phenotypic patterns provided by different authors for this species were contradictory. Recently, a new species of this genus, *Virgibacillus proomii*, was described (Heyndrickx *et al.*, 1999). Seemingly, strains of this species are variable for the VP reaction and nitrate reduction.

The species of the *Bacillus cereus* group are closely related. However, they can be differentiated on the basis of several morphological features and susceptibility to antibiotics (Claus & Berkeley, 1986; Priest *et al.*, 1988). Recently, two new species of this group have been proposed: *Bacillus pseudomycoides* (Nakamura, 1998) and *Bacillus weihenstephanensis* (Lechner *et al.*, 1998).

Group III (obligate aerobes that do not hydrolyse starch) includes species belonging to the genera *Bacillus*, *Brevibacillus* and *Aneurinibacillus*. Gelatin hydrolysis and swollen sporangia are the most effective tests for further differentiation of these micro-organisms.

Subgroups III.A and III.C (sporangia are not swollen) include the ubiquitous species *Bacillus pumilus* and *Bacillus badius*, a psychrophilic species, *Bacillus insolitus*, and two rather fastidious species, *Bacillus fastidiosus* (Claus & Berkeley, 1986) and *Bacillus sporothermodurans* (Pettersson *et al.*, 1996). *Bacillus insolitus*, together with *Bacillus globisporus*, ‘*Bacillus aminovorans*’ and *Bacillus marinus*, forms a separate phylogenetic group (Rüger, 1983). The phenotypic properties of *Bacillus marinus* and ‘*Bacillus aminovorans*’ were poorly described, so they are not represented in the key.

Swelling of the sporangium is a character peculiar to the genera *Brevibacillus* and *Aneurinibacillus*. However, in some strains of *Aneurinibacillus*, the sporangium can be only slightly swollen or not swollen at all (Heyndrickx *et al.*, 1997). *Bacillus galactophilus* was reclassified as *Brevibacillus agri* (Shida *et al.*, 1994). Subgroups III.B and III.D include thermophiles (*Bacillus thermocloaceae*, *Bacillus pallidus*, *Aneurinibacillus thermoaerophilus*), psychrophiles (*Bacillus psychrophilus*, *Bacillus globisporus*), *Bacillus sphaericus* with the closely related species (or subspecies) *Bacillus fusiformis*, and *Bacillus chitinolyticus* (Kuroshima *et al.*, 1996). Strains of *Bacillus sphaericus* could be also distinguished by resistance to streptomycin (16 µg ml⁻¹) (Priest *et al.*, 1988) or 30 µg per disk (Reva *et al.*, 1995). According to a description of *Bacillus thermosphaericus*, this species cannot convincingly be distinguished phenotypically from *Bacillus thermocloaceae* (Andersson *et al.*, 1995).

Group IV encompasses obligatory aerobic, starch-hydrolysing bacilli. This group includes species of the genera *Bacillus*, *Paenibacillus* and *Aneurinibacillus*. Nitrate reduction and the presence of swollen sporangia were the most discriminative test markers for further subdivision of this group. The central species of the group is *Bacillus subtilis*, a ubiquitous micro-organism and type species of the genus *Bacillus*. Four new species similar to *Bacillus subtilis* have been proposed: *Bacillus amyloliquefaciens* (Priest *et al.*, 1987), *Bacillus atrophaeus* (Nakamura, 1989), *Bacillus mojaviensis* (Roberts *et al.*, 1994) and *Bacillus vallis-mortis* (Roberts *et al.*, 1996). Approximately 90% of *Bacillus subtilis* strains utilized citrate and 80% of

Table 3. Index of species referred to in this paper with their key addresses

Species	Address	Reference
<i>Aneurinibacillus aneurinilyticus</i>	III.D.2.ii	Heyndrickx <i>et al.</i> (1997)
<i>Aneurinibacillus migulanus</i>	III.D.2.i	Heyndrickx <i>et al.</i> (1997)
<i>Aneurinibacillus thermoaerophilus</i>	III.B.1.ii	Meier-Staufer <i>et al.</i> (1996)
<i>Bacillus amyloliquefaciens</i>	IV.A.4	Priest <i>et al.</i> (1987)
<i>Bacillus azotoformans</i>	III.D.4.ii	Seldin <i>et al.</i> (1984)
<i>Bacillus badius</i>	III.A.2, III.C.1	Claus & Berkeley (1986)
<i>Bacillus brevis</i>	see <i>Brevibacillus</i>	Shida <i>et al.</i> (1996)
<i>Bacillus carboniphilus</i>	IV.C.4.ii	Fujita <i>et al.</i> (1996)
' <i>Bacillus carotarum</i> '	III.A.4	Priest <i>et al.</i> (1988)
' <i>Bacillus cascainensis</i> '	III.A.3	Priest <i>et al.</i> (1988)
<i>Bacillus cereus</i>	II.B.1.i, I.A.4	Claus & Berkeley (1986)
<i>Bacillus chitinolyticus</i>	III.B.2.iii	Kuroshima <i>et al.</i> (1996)
<i>Bacillus circulans</i>	II.C.1.i	Claus & Berkeley (1986)
<i>Bacillus coagulans</i>	II.B.4	Claus & Berkeley (1986)
<i>Bacillus edaphicus</i>	IV.C.3.i	Shelobolina <i>et al.</i> (1997)
<i>Bacillus ehimensis</i>	IV.B.1.ii	Kuroshima <i>et al.</i> (1996)
' <i>Bacillus endophyticus</i> '	III.C.4.i	Reva <i>et al.</i> (2001)
<i>Bacillus fastidiosus</i>	III.C.2.i	Claus & Berkeley (1986)
<i>Bacillus firmus</i>	IV.A.3	Claus & Berkeley (1986)
<i>Bacillus flexus</i>	IV.C.2.ii	Priest <i>et al.</i> (1988)
<i>Bacillus fusiformis</i>	III.B.3.i	Priest <i>et al.</i> (1988)
<i>Bacillus globisporus</i>	III.B.3.iv	Rüger (1983)
<i>Bacillus halodurans</i>	II.C.1.ii	Nielsen <i>et al.</i> (1995)
<i>Bacillus horti</i>	IV.B.2.i.β	Yumoto <i>et al.</i> (1998)
<i>Bacillus insolitus</i>	III.C.4.ii, IV.C.1	Rüger (1983)
<i>Bacillus kaustophilus</i>	see <i>Geobacillus</i>	Nazina <i>et al.</i> (2001)
<i>Bacillus laterosporus</i>	see <i>Brevibacillus</i>	Shida <i>et al.</i> (1996)
<i>Bacillus lentus</i>	IV.C.3.iv	Claus & Berkeley (1986)
<i>Bacillus licheniformis</i>	II.B.1.ii	Claus & Berkeley (1986)
<i>Bacillus megaterium</i>	IV.C.2.i	Claus & Berkeley (1986)
<i>Bacillus mucilaginosus</i>	IV.C.3.iii	Shelobolina <i>et al.</i> (1997)
<i>Bacillus naganoensis</i>	IV.D.4.iii	Tomimura <i>et al.</i> (1990)
<i>Bacillus niacini</i>	I.B.4	Nagel & Andersen (1991)
<i>Bacillus oleronius</i>	IV.D.4.ii	Kuhnigk <i>et al.</i> (1995)
<i>Bacillus pallidus</i>	III.D.1.i	Scholz <i>et al.</i> (1987)
<i>Bacillus pantothenicus</i>	see <i>Virgibacillus</i>	Heyndrickx <i>et al.</i> (1998)
<i>Bacillus psychrophilus</i>	III.B.4.i	Priest <i>et al.</i> (1988)
<i>Bacillus psychrosaccharolyticus</i>	IV.C.4.i	Priest <i>et al.</i> (1988)
<i>Bacillus pumilus</i>	III.A.1	Claus & Berkeley (1986)
' <i>Bacillus racemilacticus</i> '	I.A.3	Spring <i>et al.</i> (1996)
<i>Bacillus silvestris</i>	III.D.3.iii	Rheims <i>et al.</i> (1999)
<i>Bacillus simplex</i>	IV.A.1	Priest <i>et al.</i> (1988)
<i>Bacillus smithii</i>	II.C.2	Nakamura <i>et al.</i> (1988)
<i>Bacillus sphaericus</i>	III.B.3.iii	Claus & Berkeley (1986)
<i>Bacillus sporothermodurans</i>	III.C.2.ii	Pettersson <i>et al.</i> (1996)
<i>Bacillus stearothermophilus</i>	see <i>Geobacillus</i>	Nazina <i>et al.</i> (2001)
<i>Bacillus subtilis</i>	IV.A.2	Claus & Berkeley (1986)
<i>Bacillus thermocatenulatus</i>	see <i>Geobacillus</i>	Nazina <i>et al.</i> (2001)
<i>Bacillus thermocloaceae</i>	III.D.1.ii	Demharther & Hensel (1989)
<i>Bacillus thermodenitrificans</i>	see <i>Geobacillus</i>	Nazina <i>et al.</i> (2001)
<i>Bacillus thermoglucosidasius</i>	see <i>Geobacillus</i>	Nazina <i>et al.</i> (2001)
<i>Brevibacillus agri</i>	III.B.1.i	Nakamura (1993)
<i>Brevibacillus borstelensis</i>	III.B.4.iv	Shida <i>et al.</i> (1995)
<i>Brevibacillus brevis</i>	III.B.4.ii	Claus & Berkeley (1986)

Table 3 (cont.)

Species	Address	Reference
<i>Brevibacillus centrosporus</i>	III.B.2.ii, III.D.4.i, III.D.3.ii	Nakamura (1993)
<i>Brevibacillus choshinensis</i>	III.D.3.i	Takagi <i>et al.</i> (1993)
<i>Brevibacillus formosus</i>	III.B.2.i	Shida <i>et al.</i> (1995)
<i>Brevibacillus laterosporus</i>	I.B.1.ii	Claus & Berkeley (1986)
<i>Brevibacillus parabrevis</i>	III.B.1.iv	Takagi <i>et al.</i> (1993)
<i>Brevibacillus reuszeri</i>	III.D.3.iv	Shida <i>et al.</i> (1995)
<i>Geobacillus kaustophilus</i>	IV.B.1.i	Priest <i>et al.</i> (1988)
<i>Geobacillus stearothermophilus</i>	II.C.1.iv	Claus & Berkeley (1986)
<i>Geobacillus thermocatenulatus</i>	II.D.1.ii	Golovacheva <i>et al.</i> (1975)
<i>Geobacillus thermodenitrificans</i>	II.D.1.iv	Claus & Berkeley (1986)
<i>Geobacillus thermoglucosidasius</i>	IV.D.4.i	Suzuki <i>et al.</i> (1983)
<i>Paenibacillus alginolyticus</i>	IV.D.1	Nakamura (1987)
<i>Paenibacillus alvei</i>	II.A.1	Claus & Berkeley (1986)
<i>Paenibacillus amylolyticus</i>	II.D.3.iv	Nakamura (1984)
<i>Paenibacillus apiarius</i>	II.D.4.i	Nakamura (1996)
<i>Paenibacillus azotofixans</i>	I.A.1	Shida <i>et al.</i> (1997a)
<i>Paenibacillus borealis</i>	I.B.3.i	Elo <i>et al.</i> (2001)
<i>Paenibacillus chibensis</i>	IV.B.1.iii	Shida <i>et al.</i> (1997b)
<i>Paenibacillus chondroitinus</i>	IV.D.3.i	Nakamura (1987)
<i>Paenibacillus curdlanolyticus</i>	IV.B.2.ii	Kanzawa <i>et al.</i> (1995)
<i>Paenibacillus dendritiformis</i>	II.C.4	Tcherpakov <i>et al.</i> (1999)
<i>Paenibacillus glucanolyticus</i>	II.D.3.ii	Alexander & Priest (1989)
<i>Paenibacillus illinoisensis</i>	II.C.1.iii	Shida <i>et al.</i> (1997b)
<i>Paenibacillus kobensis</i>	IV.B.2.i.α	Shida <i>et al.</i> (1997a)
<i>Paenibacillus koreensis</i>	II.D.1.i	Chung <i>et al.</i> (2000)
<i>Paenibacillus larvae</i> subsp. <i>larvae</i>	I.B.3.ii	Heyndrickx <i>et al.</i> (1996a)
<i>Paenibacillus larvae</i> subsp. <i>pulvificiens</i>	I.B.1.i	Heyndrickx <i>et al.</i> (1996a)
<i>Paenibacillus lautus</i>	II.D.4.ii	Heyndrickx <i>et al.</i> (1996b)
<i>Paenibacillus macerans</i>	II.D.1.iii	Claus & Berkeley (1986)
<i>Paenibacillus macquariensis</i>	II.C.3.iv	Priest <i>et al.</i> (1988)
<i>Paenibacillus pabuli</i>	II.C.3.ii	Nakamura (1984)
<i>Paenibacillus peoriae</i>	II.A.2	Montefusco <i>et al.</i> (1993)
<i>Paenibacillus polymyxa</i>	II.B.2	Claus & Berkeley (1986)
<i>Paenibacillus thiaminolyticus</i>	II.D.2	Nakamura (1990)
<i>Paenibacillus validus</i>	IV.D.3.ii	Nakamura (1984)
<i>Virgibacillus pantothenicus</i>	II.C.3.i, II.D.3.i	Heyndrickx <i>et al.</i> (1999)
<i>Virgibacillus proomii</i>	II.C.3.iii	Heyndrickx <i>et al.</i> (1999)

Bacillus amyloliquefaciens strains were negative by this test. Identification of these species by citrate utilization (as recommended in the key) is therefore rather probabilistic.

Another ubiquitous species is *Bacillus megaterium*, a representative of subgroup IV.C. Species such as *Bacillus flexus* and *Bacillus simplex* are often considered as subspecies of *Bacillus megaterium* (Priest *et al.*, 1988). The newly described species *Bacillus mucilaginosus* and *Bacillus edaphicus* were shown to be genetically close to *Paenibacillus* species, so they could probably be considered as representatives of this genus (Shelobolina *et al.*, 1997).

The developed key was used for the identification of 100 bacillus strains from the UCM including some

type strains and reference strains with known taxonomic positions and some newly isolated strains. The majority of the type and reference strains shown in Table 1 were identified correctly by the key, except for *Paenibacillus polymyxa* UCM B-5760 (the spores did not develop in swollen sporangia and anaerobic growth did not occur) and *Virgibacillus pantothenicus* UCM B-5907 (starch hydrolysis was negative, anaerobic growth, nitrate reduction and mannitol fermentation were positive). The strain '*Bacillus entomocidus* var. *subtoxicus*' UCM B-5900 was identified as a member of the *Bacillus cereus* group (in the ATCC catalogue, this strain is listed as *Bacillus thuringiensis*) and '*Bacillus longissimus*' UCM B-5880 was identified as *Bacillus subtilis* (in the VKM catalogue, this strain is listed as *Bacillus* sp.). Strain UCM B-5656, identified

previously as *Bacillus cereus*, was identified by the key as *Bacillus licheniformis*. Strains UCM B-5044, 103D, 106D, 107D, 108D, 109D, 111D, 115D and 119D were isolated from the inner tissues of plants and were identified by the key as *Bacillus subtilis*. However, RAPD typing and 16S rRNA sequencing showed that only UCM 119D is a strain of *Bacillus subtilis* and all the other endophytic strains belong to *Bacillus amylo-liquefaciens*. Another group of endophytic strains, UCM B-5908, B-5910, B-5911 and 9DT, were characterized by a very specific pattern of phenotypic properties that distinguished them from all other known *Bacillus* species. Analysis of the genotype of these strains confirmed their specificity and a new species, '*Bacillus endophyticus*', which is represented in the key in cell III.C.4.i, has recently been proposed (Reva *et al.*, 2001). Two strains, 1r and 2r, newly isolated from the soil near the destroyed reactor of the Chernobyl nuclear power station, were identified respectively as *Bacillus licheniformis* and *Bacillus pumilus*. RAPD typing and ribotyping confirmed the results of identification by the key.

A new species, '*Paenibacillus granivorans*', has recently been proposed (Van der Maarel *et al.*, 2000). The phenotypic characters provided do not distinguish this taxon from *Paenibacillus curdlanolyticus* (Kanzawa *et al.*, 1995) according to our key. Unfortunately, the authors did not include this species among the reference strains in their genetic study, so it is difficult to consider the relationship between these micro-organisms.

The proposed key for identification is quite practical and convenient to use, since it permits species identification on the basis of a few simple tests. For confirmation, several additional tests may be needed. An index of species of aerobic spore-forming bacteria with their addresses in the key (see Fig. 1) and references for more details is presented in Table 3.

New species are frequently described using sophisticated methods of genetics and biochemistry, but their phenotype is poorly described. Moreover, different laboratories use different sets of descriptive tests. We therefore could not include the following species in our proposed key, since they had not been characterized by the tests used in the key: '*Bacillus aminovorans*' and *Bacillus marinus* (Rüger, 1983), '*Bacillus flavothermus*' (Heinen *et al.*, 1982), '*Bacillus macroides*' (Claus & Berkeley, 1986), *Bacillus methanolicus* (Arfman *et al.*, 1992), several moderately alkaliphilic bacilli (*Bacillus clausii*, *Bacillus gibsonii*, *Bacillus halmaphilus*, *Bacillus horikoshii*, *Bacillus pseudocalcaliphilus* and *Bacillus pseudofirmus*; Nielsen *et al.*, 1995), *Brevibacillus thermoruber* (Manachini *et al.*, 1985), *Geobacillus subterraneus* and *Geobacillus uzenensis* (Nazina *et al.*, 2001) and *Geobacillus thermoleovorans* (Zarilla & Perry, 1987).

The data in Table 2 are helpful for selecting useful and effective tests for the description of newly isolated

strains and species. The identification key discussed above is represented in interactive mode on a website (<http://www/imv.kiev.ua/key/>).

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