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RESEARCH ARTICLE

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Characterization of *Bacillus* strains of marine origin

Summary A total of twenty aerobic endospore-forming bacilli, isolated from marine invertebrates and sea water of different areas of the Pacific Ocean, were taxonomically characterized. Most of the bacilli (11 strains) of marine origin belonged to the species *Bacillus subtilis*, according to their phenotypic characteristics, antibiotic susceptibility profiles, and fatty acids patterns. A group of four alkaliphilic strains formed a separate cluster that was tentatively classified as *B. horti.* One isolate, KMM 1717, associated with a sponge from the Coral Sea was identified as *B. pumilus.* Two strains, *Bacillus* KMM 1916 and KMM 1918, showed antibiotic sensitivity profiles similar to *B. licheniformis*, but they had a distinct fatty acid composition and peculiar phenotypic traits. The taxonomic affiliation of KMM 1810 and KMM 1763 remained unclear since their fatty acid composition and antibiotic sensitivity patterns were not resembled with none of these obtained for *Bacillus* strains.

Key words *Bacillus* spp. · Phenotypic characterization · Fatty acid analysis · Marine microbiology

Introduction

The genus *Bacillus* comprised a phylogenetically and phenotypically heterogeneous group of species. Recently, the systematic of the *Bacillus* group has been widely modified. On the basis of extensive studies of the small-subunit ribosomal RNA sequences, the species of the genus *Bacillus* were split into four distinct clusters and several ungrouped species, such as: group 1 (*Bacillus* sensu stricto*)*, which includes *B. subtilis*, the type species of the genus, and 27 other species [1]; group 2 includes the round-spore-forming bacilli, together with some asporogenous taxa (the genera *Caryophanon*, *Exiguobacterium*, *Kurthia*, and *Planococcus*); the group constitutes a distinct cluster, only remotely related to *B. subtilis* [6]; group 3, with ten representatives, comprises *B. polymyxa* and *B. macerans*, which have been reclassified in the new genus *Paenibacillus* [2]; and group 4, with strains classified into two newly created genera, *Aneurinibacillus* and *Brevibacillus* [25]. Besides, a new genus, *Virgibacillus*, was recently created to accommodate former *B. pantothenticus* [11]. Finally, several newly isolated *Bacillus* species have been described, including *B. mojavensis* and *B. vallismortis* [23, 24], *B. ehimensis* and *B. chitinolyticus* [18], *B. infernus* [4], *B. carboniphilus* [7], and *B. horti* [30].

Few publications are devoted to the study of the *Bacillus* species isolated from the marine environment. Due to their ubiquity and capability to survive under adverse conditions, heterotrophic *Bacillus* strains are hardly considered to be species of certain habitats [5]. A heterogeneous group of moderately halophilic bacteria, which comprises *B. salexigens*, and three species of the new genus *Halobacillus*, *H. halophilus*, *H. litoralis*, and *H. trueperi* [9, 26, 29] may be differentiated by their ability to grow at 10 to 20% of total salts and the possession of an unusual type of murein. Species of *B. marinus*, *B. badius B. subtilis*, *B. cereus*, *B. licheniformis*, *B. firmus*, and *B. lentus* were often isolated from marine habitats [3, 5, 22]. Our recent studies on marine bacilli [12] showed that strains of *B. marinus*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, *B. cereus*, and *B. mycoides* are common inhabitants of the Pacific Ocean habitat.

A group of marine *Bacillus* strains from the Collection of Marine Microorganisms (KMM) of the Pacific Institute of Bio-organic Chemistry (Vladivostok, Russia) has been taxonomically studied in view of their ability to produce biologically active compounds [13, 15]. A few bacilli of marine origin have been reported to produce unusual metabolites different from those isolated from terrestrial bacteria [14]. These metabolites include an antibiotic, 3-amino-3-deoxy-D-glucose [8], a new glucanase [21], and cyclic acylpeptides [10, 28]. The

aim of the present paper is to clarify the taxonomic affiliation of several *Bacillus* strains of marine origin from KMM that produced a number of physiologically active compounds.

Materials and methods

Bacterial strains and cultivation During the 7th research cruise of R/V «Akademic Oparin», in June–November 1988, samples of sea water and marine animals were collected by scuba-diving at a depth of 6–12 m in the Sea of Japan, Okhotsk Sea, and Coral Sea of the Pacific Ocean. Isolates studied are listed in Table 1. The isolation procedure, and media of cultivation have been described elsewhere [12]. The *Bacillus* strains were grown also on Nutrient Agar (NA) at 28°C for 24–48 h. The strains were isolated by plating and phase-contrast microscopy, and were maintained both as lyophilized cultures and on semisolid NA in tubes at 4°C.

Phenotypic characterization For the phenotypic characterization studies the following reference strains were used: *B. licheniformis* KMM 670^T (= ATCC 14580^T), *Bacillus pumilus* KMM 683^T (= ATCC 7061^T), *Bacillus subtilis* KMM 676^T (=ATCC 6051^T), and *B. horti* JCM 9943^T. Strains isolated in the present study were characterized by conventional microbiological methods [6, 13], using API 20E and API 50CH systems[19] and, morphology of vegetative cells and sporangia, and shape and position of spores. In addition, the following characteristics were studied: nitrate reduction test; anaerobic growth; gas production from nitrate and glucose; degradation of starch, urea, casein, Tween-20, Tween-40, Tween-80, gelatin, chitin and agar; acid production from D-arabinose, D-xylose, D-glucose, and mannitol; utilization citrate, and propionate; growth at 4°, 10°, 25°, 30°, 37°, 40°, 50°, 55°C; and NaCl requirement (0, 1, 3, 7, 10, 12, 15%). Growth at different pH (5.7–11.5) was detected on the medium (B) that contained (wt/vol): 0.2% Bacto-peptone (Difco), 0.2% casein hydrolysate (Merck), 0.2% yeast extract (Difco), 0.1% glucose, 0.02% KH_2PO_4, 0,005% $MgSO_4 \cdot 7H_2O$, 1.5% Bacto-agar (Difco), 50% (vol/vol) of natural seawater and 50% distilled water. The pH was adjusted with 10 M NaOH.

Cluster analysis were performed using STATISTICA software (rel. 4.3B, StatSoft 1993) for Windows. An unweighted pair group average method was used for cluster analysis, and a dendrogram was drawn by using a percentage disagreement method.

Antibiotic sensitivity Sensitivity to antibiotic was determined by using the routine diffusion plate technique. Cultures were grown overnight on the nutrient medium B at 28°C, and were used to prepare suspensions with optical density of 0.5 McFarland Standard ($1,5 \times 10^8$ cells per ml). A 0.1-ml portion of suspension was plated onto agar, and disks containing antibiotics were placed onto the surface of the medium. After overnight incubation at 30°C the diameters of the zones of growth inhibition were measured. The following antibiotics were used (μg/disk): oleandomycin (15 μg), oxacillin (10 μg), ristomycin (30 μg), gentamicin (10 μg), chloramphenicol (30 μg), ampicillin (10 μg), erythromycin (15 μg), kanamycin (30 μg), carbenicillin (25 μg), and benzylpenicillin (10 U).

DNA base composition and fatty acid analysis DNA was isolated from the cells grown overnight in NA. The G + C content of the DNA was determined by the method of Marmur and Doty [20]. Fatty acid (FA) composition was essentially studied as described by Svetashev et al. [27]. Branched unsaturated FA were identified by equivalent chain length (ECL) from Kaneda [16, 17].

Results and Discussion

We selected for taxonomic purposes twenty bacilli of marine origin which were isolated mainly from sponges, ascidian, crabs, and seawater samples collected from the Sea of Japan, the Sea of Okhotsk, and the Coral Sea of the Pacific Ocean. All strains possessed typical cellular and colonial morphologies, physiological, biochemical, and nutritional features that resembled them to *Bacillus* spp. sensu stricto. The organisms were motile and produced oval endospores located at subterminal or central positions in the sporangia. Other phenotypic characteristics of the strains studied are included in the Table 1. Antibiotic sensitivity tests revealed profile patterns for strains KMM 1916 and KMM 1918 similar to the *B. licheniformis* pattern, though the latter species was sensitive to lincomycin. A group of isolates of *B. pumilus* and *B. horti*-like phenotypes were distinguishable from the other species by their high sensitivity to penicillin-like antibiotics, different from all strains of *B. subtilis*. The API Biotype and other phenotypic features revealed that the majority of the strains tested (11 out of 19) belonged to *B. subtilis*. Strains KMM 1763, KMM 1717, KMM 1918, and 1916 could not be identified as any of well-defined phenotypes included in API database.

A comparative analysis of cellular FA supported the results of tentative identification and allowed to perform more accurate discrimination of environmental isolates (Fig. 1). The later exhibited characteristic FA patterns useful for species discrimination. The FA profiles of the major group (two strains, KMM 441 and KMM 444, out of eleven of *B. subtilis*-like phenotype, are not included into Fig. 1) indicated similarity to the type strain of *B. subtilis* pattern. The principal FA found in marine strains were 12-methyltetradecanoic (anteiso C15:0) and 13-methyltetradecanoic (iso C15:0), 14-methylhexadecanoic (anteiso C17:0), 15-methylhexadecanoic (iso C17:0), and 14-methylpentadecanoic (iso C16:0). FA profiles of KMM 1717 resembled that of type strain of *B. pumilus*, which is in consistence to similar phenotypic features of both strains. Therefore the strain KMM 1717 belongs to *B. pumilus*. Four other alkaliphilic marine

Table 1 Phenotypic comparison of *Bacillus* species of marine origin and some other related bacilli species

Characteristic	*B. subtilis* ATCC 6051^T	Marine isolates of *B. subtilis*	*B. pumilus* KMM 683^T	*B. pumilus* KMM 1717	*B. hortii* JCM 9943^T	Marine isolates of *B. horti*	*Bacillus* sp. KMM 1810	*Bacillus* sp. KMM 1918	*Bacillus* sp. KMM 1916	*Bacillus* sp. KMM 1763
Oxidase activity	+[a]	90[b]	–	–	+	+	–	+	–	–
Max. growth temp. (°C)	50	50–55	50	50	40	45	45	45	45	45
Min. growth temp. (°C)	10	15	10	15	10	10	10	15	15	15
Starch hydrolysis	+	36	–	–	+	100	–	–	–	+
Casein	+	0	+	–	+	0	+	–	+	–
Tween-20	+	45	–	–	–	100	–	–	–	–
Tween-40	–	27	+	–	–	0	–	+	–	+
Tween-80	–	9	–	–	–	0	–	–	–	–
Growth at:										
pH 5.7–7.5	+	100	+	–	+	75/100	+	+	+	+
pH 9.5–11.5	–	100	–	+	+	100	+	+	+	+
Growth in NaCl:										
0%–7%	+	100	+	+	+	100	+	+	+	+
10%	+	55	+	+	+	100	+	+	–	+
15%	–	0	–	–	–	100	+	–	–	+
Acid produced from:										
D-Glucose	+	100	+	+	+	100	+	+	+	+
D-Xylose	+	73	+	+	+	100	+	–	–	–
D-Arabinose	+	18	+	+	–	100	+	–	–	–
Lactose	–	0	–	–	–	0	–	–	–	–
D-Mannitol	+	100	+	+	+	100	+	+	+	–
Utilization of:										
Citrate	+	82	+	+	+	100	+	+	+	–
Antibiotics susceptibility[c]:										
Oxacillin, ampicillin, carbenicillin, benzyl-penicillin	21–28	20–33	30–42	28–35	30–36	36–54	38–50	0-18	0-18	14–16
Chloramphenicol	27	24–31	20	25	20–22	22–24	22	20	20	15
Lincomycin	18	0-13	0	0	0	0	25	0	0	15
Oleandomycin	22	20–24	20	25	22–24	26–30	32	14	14	25
Habitat		Sea water, sponges, ascidians, corals		Sponge, coral sea	Soil	Sea water, crab *Callinectes sapidus*, sponges	Sponge *Lantriculia* sp.	Okhotsk Sea, crab *Paralithodes camtschatica*	Okhotsk Sea, water	Coral Sea, sponge, *Phylospongidae*
GC content (mol%)	43	40–43	42	41	40–41	39–40	42	41	40	31

[a]+, positive reaction. –, negative reaction. ND, not determined.
[b]values are the percentages of strains that exhibit positive reactions; n = 11 for *B. subtilis* (KMM 1788, KMM 1786, KMM 1765, KMM 1745, KMM 1847, KMM 1917, KMM 441, KMM 444, KMM 1756, KMM 456, KMM 446); and n = 4 for *B. horti* (KMM 1618, KMM 1669, KMM 1751, KMM 1797).
[c]range of the mean diameter of inhibited zone, mm.

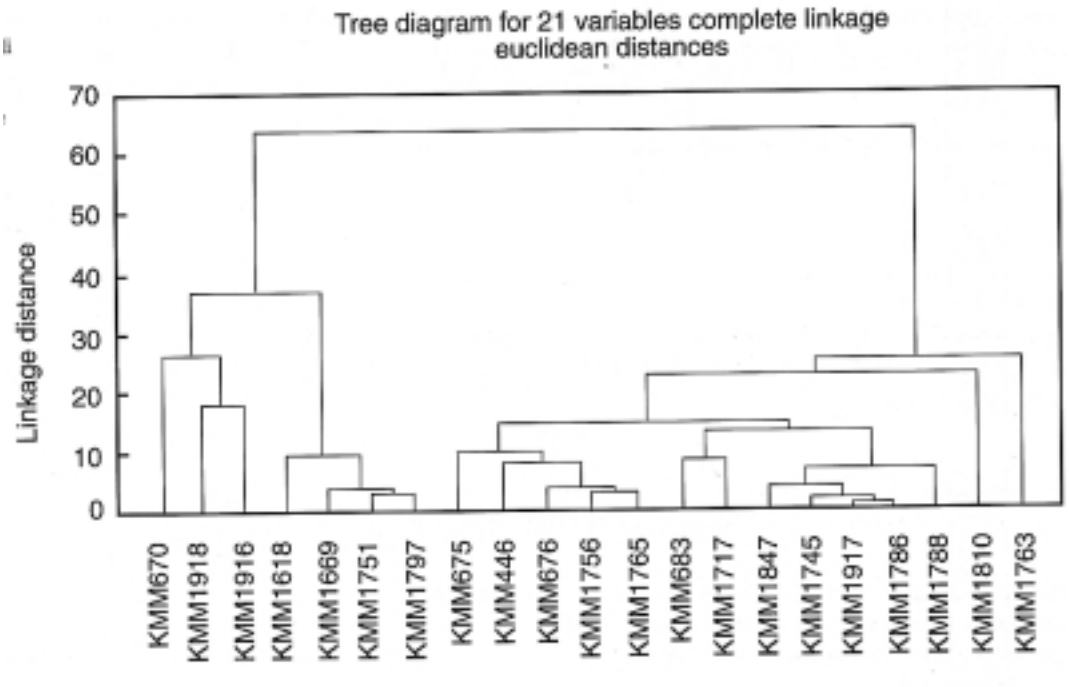

Fig. 1 Dendrogram showing the clustering of 18 marine bacilli strains and type strains *Bacillus licheniformis* KMM 670^T (ATCC 14580^T), *Bacillus subtilis* KMM 675^T (ATCC 6051^T), *Bacillus pumilus* KMM 683^T based on fatty acids profiles. The dendrogram was derived from Euclidean distances

isolates, KMM 1618, KMM 1669, KMM 1751, KMM 1797, had FA composition almost identical to that of alkaliphilic *B. horti* [30] and formed a cluster at a Euclidean distance of about 10, a level recently proposed for species delineation [11]. Their predominant FA were iso C15:0 up to 42%, and anteiso C15:0 up to 30%. This results suggested that four marine isolates belong to *B. horti*, in spite the fact that they had a few differences in biochemical traits. Isolates KMM 1916 and KMM 1918 formed two distinct clusters and might represent novel species.

Among the numerous *Bacillus* species, only species of *B. badius B. subtilis, B. cereus, B. lichenifirmis, B. firmus, B. pumilus, B. mycoides,* and *B. lentus* were reported to have been detected from marine environments. There are true marine species, such as *B. marinus, B. salexigens, B. dipsosauri* [9], and the species of the newly created genus *Halobacillus (H. halophilus, H. litoralis,* and *H. trueperi*) that require NaCl ions for growth [26]. Nevertheless, according to our previous [12] and present observations, the strains of *B. subtilis* and *B. pumilus* were the most abundant among those associated with marine sponges, ascidians, soft corals, and they were present in seawater as well. In addition, among the marine bacilli, the strains which belonged to *B. horti* and some other distinct phenotypes have been identified. All of the strains studied were able to utilize a wide range of organic compounds, were halotolerant and alkalitolerant, which may reflect their great metabolic flexibility.

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References

1. Ash C, Farrow JAE, Wallbanks S, Collins MD (1991) Phylogenetic heterogeneity of the genus *Ballicus* revealed by comparative analysis of the small-subunit-ribosomal RNA. Lett Appl Microbiol 13:202–206
2. Ash C, Priest FG, Collins D (1993) Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Antonie van Leeuwenhoek 64:253–260
3. Boeye H, Aerts M (1976) Numerical taxonomy of *Bacillus* isolates from North Sea sediments. Int J Syst Bacteriol 26:427–441
4. Boone DR, Liu Y, Zhao Z-Ju, Balkwill DL, Drake GR, Stevens TO, Aldrich HC (1995) *Bacillus infernus* sp. nov. an Fe(III)- and Mn(IV)-reducing anaerobe from the deep terrestrial subsurface. Int J Syst Bacteriol 45:441–448
5. Claus D, Berkeley RCW (1986) Genus *Bacillus,* Cohn 1872. In: Sneath PHA, Mair NS, Sharpe ME, Holt JG (eds) Bergey's Manual of Systematic Bacteriology. Vol. 2. Baltimore: The Williams and Wilkins Co, pp 1105–1139
6. Farrow JAE, Wallbanks S, Collins MD (1994) Phylogenetic interrelationship of round-spore-forming bacilli containing cell walls based on lysine and the non-spore-forming genera *Caryophanon, Exiguobacterium, Kurthia, and Planococcus.* Int J Syst Bacteriol 44:74–82
7. Fujita T, Shida O, Takagi H, Kunugita K, Pankrushina AN, Matsuhashi M (1996) Description of *Bacillus carboniphilus* sp. nov. Int J Syst Bacteriol 46:116-118
8. Fusetani N, Ejima D, Matsunaga S, Hatsumoto K, Itagaki K, Akagi Y, Taga N, Suzuki K (1987). 3-Amino-3-deoxy-D-glucose: an antibiotic produced by deep-sea bacterium. Experientia 43:464–465
9. Garabito MJ, Arahal DR, Mellado E, Marquez MC, Ventosa A (1997) *Bacillus salexigens* sp. nov., a new moderately halophilic *Bacillus* species. Int J Syst Bacteriol 47:735–741
10. Gerard J, Haden P, Kelly MT, Andersen RJ (1996) Loloatin B, a cyclic decapeptide antibiotic produced in culture by a tropical marine bacterium. Tetrahedron Lett 37:7201–7204
11. Heyndrickx M, Lebbe L, Kersters K, De Vos P, Forsyth G, Logan NA (1998) *Virgibacillus*: a new genus to accommodate *Bacillus pantothenticus* (Proom and Knight 1950). Emended description of *Virgibacillus pantothenticus*. Int J Syst Bacteriol 48:99–106
12. Ivanova EP, Mikhailov VV, Andreev LA (1992) Marine bacilli and some

approaches to their identification. Mikrobiol Zhurnal 54:27–33 (in Russian)
13. Ivanova EP, Nicolau DV, Yumoto N, Taguchi T, Okamoto K, Tatsu Y, Yoshikawa S (1998) Impact of the conditions of cultivation and adsorption on antimicrobial activity of marine bacteria. Mar Biol 130:545–551
14. Jensen PR, Fenical W (1994) Strategies for the discovery of secondary metabolites from marine bacteria: ecological perspectives. Annu Rev Microbiol 48:559-584
15. Jensen PR, Harvel CD, Wirtz K, Fenical W (1996) Antimicrobial activity of extracts of Caribbean gorgonian corals. Mar Biol 125:411–419
16. Kaneda T (1977) Fatty acids of the genus *Bacillus*: an example of branched-chain preference. Bacteriol Rev 41:391–418
17. Kaneda T (1991) Iso- and anteiso fatty acids in bacteria: biosynthesis, function and taxonomic significance. Microbiol Rev 55:288–302
18. Kuroshima, KI, Sakane T, Takata R, Yokota A (1996) *Bacillus ehimensis* sp. nov and *Bacillus chitinolyticus* sp. nov., new chitinolytic members of the genus *Bacillus.* Int J Syst Bacteriol 46:76–80
19. Logan NA, Berkeley RCW (1984) Identification of *Bacillus* strains using the API system. J Gen Microbiol 130:1871–1882
20. Marmur J, Doty P (1962) Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J Mol Biol 5:109–118
21. Okami Y, Kurasawa S, Hirose Y (1980) A new glucanase produced by marine *Bacillus*. Agric Biol Chem 44:1191–1192
22. Ortigosa M, Garay E, Pujalte M-J (1997) Gram-positive bacteria of marine origin: a numerical taxonomic study on Mediterranean isolates. Microbiología SEM 13:543–462
23. Roberts MS, Nakamura LK, Cohan FM (1994) *Bacillus mojavensis* sp. nov., distinguishable from *Bacillus subtilis* by sexual isolation, divergence in DNA sequences, and differences in fatty acid composition. Int J Syst Bacteriol 44:256–264
24. Roberts MS, Nakamura LK, Cohan FM (1996) *Bacillus vallismortis* sp. nov., a close relative of *Bacillus subtilis* isolated from soil in Death Valley, California. Int J Syst Bactriol 46:470–475
25. Shida O, Takagi H, Kadowaki K, Komagata K (1996) Proposal for two new genera, *Brevibacillus* gen nov. and *Aneurinibacillus* gen nov. Int J Syst Bacteriol 46:939–946
26. Spring S, Ludwig W, Marquez MC, Ventosa A, Schleifer K-H (1996) *Halobacillus* gen. nov., with descriptions of *Halobacillus litoralis* sp. nov. and *Halobacillus trueperi* sp. nov., and transfer of *Sporosarcina halophila* to *Halobacillus halophilus* comb. nov. Int J Syst Bacteriol 46:492–496
27. Svetashev VI, Vysotskii MV, Ivanova EP, Mikhailov VV (1995) Cellular fatty acids of *Alteromonas* species. Syst Appl Bacteriol 18:37–43
28. Trischman J, Jensen RP, Fenical W (1994) Halobacillin: a cytotoxic cyclic acylpeptide of the iturin class produced by a marine *Bacillus* sp. Tetrahedron Lett 35:5571–5574
29. Ventosa A, Garcia MT, Kamekura M, Onishi H, Ruiz-Berraquero F (1989) *Bacillus halophilus* sp. nov., a moderately halophilic *Bacillus* species. System Appl Microbiol 12: 162–166
30. Yumoto I, Yamasaki K, Sawabe T, Nakano K, Kawasaki K, Ezura Y, Shinano H (1998) *Bacillus horti* sp. nov., a new Gram-negative alkaliphilic bacillus. Int J Syst Bacteriol 48:565–571