New psychrophilic and psychrotolerant *Bacillus marinus* strains from tropical and polar deep-sea sediments and emended description of the species

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In contrast to the current view that psychrophily combined with an absolute requirement for NaCl is connected with the Gram-negative cell wall type, psychrophilic and psychrotolerant, NaCl-requiring, Gram-positive bacteria have been isolated from tropical Atlantic, Arctic and Antarctic deep-sea sediments. Some of the isolates are even extremely psychrophilic, having maximum growth temperatures of 4 °C. On the basis of phenotypic characteristics, DNA base analyses, DNA–DNA hybridizations and partial and complete 16S rRNA gene sequence analyses, the strains from the three distinct geographical regions have been allocated to the obligately marine species *Bacillus marinus*. The distribution and origin of *B. marinus* are discussed and an emended description of the species is presented.

Keywords: *Bacillus marinus*, NaCl requirement, psychrophilic, polar regions, tropical deep sea

INTRODUCTION

Members of the genus *Bacillus* are ubiquitous in terrestrial and freshwater habitats and are also widely distributed in the world oceans (ZoBell & Upham, 1944; ZoBell, 1946; Denis *et al.*, 1975; Brisou *et al.*, 1978; Bonde, 1981; Rüger, 1989). Bacilli from seawater samples and marine bottom deposits are moderately halotolerant. They are able to propagate and to metabolize under marine conditions, but the vast majority are not dependent on seawater media for growth and are therefore supposed to be of terrestrial origin.

So far, only a few seawater medium-requiring *Bacillus* strains have been isolated from marine sources (Brisou & Denis, 1969; Fahmy, 1978; Boeyé & Aerts, 1976; Rüger & Koploy, 1980). *Bacillus marinus*, originally described as *Bacillus globisporus* subsp. *marinus* by Rüger & Richter (1979) and later elevated to

species rank (Rüger, 1983), proved to be dependent on sodium and potassium ions for growth and is therefore regarded as a true marine species (Rüger & Hentzschel, 1980).

Many of the marine and terrestrial environments inhabited by members of the genus *Bacillus* are permanently, seasonally or temporarily cold, with temperatures near 0 °C or below. It is not surprising, therefore, that numerous *Bacillus* strains capable of growing at low temperatures could be isolated from many different habitats (Larkin & Stokes, 1966; Laine, 1970; Abd El-Rahman, 1988). Marshall & Ohye (1966) described the cold-adapted *Bacillus macquariensis* from soils of the Antarctic Macquarie Island and Larkin & Stokes (1967) reported the taxonomy of four new *Bacillus* species able to grow at 0 °C or below, namely *Bacillus psychrosaccharolyticus*, *Bacillus insolitus*, *Bacillus globisporus* and *Bacillus psychrophilus*.

Until now, there has been no binding definition of the term psychrophilic. The most accepted definition is that given by Morita (1975), requiring growth at 0 °C or at subzero temperatures, optimum growth temperatures of 15 °C or lower and maximum growth temperatures of below 20 °C. Cold-adapted bacteria with maximum growth temperatures higher than 20 °C

Abbreviations: DAP, diaminopimelic acid; FWB, freshwater broth; MOF, marine oxidation/fermentation; SWA, seawater agar; SWB, seawater broth.

The EMBL accession number of the 16S rDNA gene sequence of *Bacillus marinus* DSM 1297^T is AJ237708.

are regarded as 'psychrotrophic' (Baross & Morita, 1978). In accordance with these definitions, all the cold-adapted bacilli mentioned above are therefore 'psychrotrophic', including those strains that were originally characterized as psychrophilic.

Instead of 'psychrotrophic', which means 'cold-eating', the term psychrotolerant seems to be more logical for organisms normally growing at temperatures above 20 °C, but also being able to grow at near 0 °C or below (Russell, 1993; Nedwell & Rutter, 1994). Therefore, the term psychrotolerant is preferred in this paper.

In contrast to the current view, that strict psychrophily plus an absolute requirement for NaCl is connected with the Gram-negative cell wall type, Rüger & Tan (1992) reported the isolation of psychrophilic, seawater-requiring, Gram-positive bacteria from the permanently cold deep-sea floor of the eastern tropical Atlantic. During two subsequent expeditions in 1987 and 1990, aerobic spore-forming bacteria with the same phenotypic characteristics as the tropical deep-sea strains have been isolated from Arctic and Antarctic bottom sediments. On the basis of DNA base analyses, DNA-DNA hybridizations and 16S rRNA gene sequencing, the strains from these three distinct geographical regions have been allocated to *B. marinus*. The distribution and origin of this species are discussed and an emended description of *B. marinus* is presented, incorporating the phenotypic characteristics of the new isolates.

METHODS

Isolation of strains. The bacteria listed in Table 1 were isolated from sediment samples during the expedition GEOTROPEX '83 with the German research vessel *Meteor* in the eastern tropical Atlantic in 1983 and during two expeditions with the German research vessel *Polarstern* in 1987 and 1990 in the Arctic and Antarctic seas, respectively. The geographical positions of sampling sites and the corrected depths were taken from Sarnthein *et al.* (1983), Tan & Rüger (1991) and Tan *et al.* (1999).

Sediment sample processing and isolation of strains were done as described previously (Rüger & Tan, 1992). All solutions and agar media were pre-chilled to 4 °C and held at this temperature on a cold tray during the whole inoculation procedure. Seawater agar (SWA) according to ZoBell (1946) was used as isolation medium, but was modified and consisted of 1.5 g Bacto Peptone, 0.3 g yeast extract, 0.01 g $\text{FePO}_4 \cdot 4\text{H}_2\text{O}$, 10.0 g Agar no. 1 (Oxoid), 750 ml seawater and 250 ml distilled water; pH 7.6. The incubation temperatures were 2 and 20 °C for the tropical strains and 4 °C for the polar strains. For isolation of *Bacillus* strains from dormant spores (strains A02712, A02720, A02800, A02819, A02735, A02736 and A02738), dilutions of sediment subsamples were heated for 10 min at 80 and 90 °C and spread onto spore germination medium II (Rüger, 1975).

Spore formation. The ability to form endospores was determined in the following test media: SWA, SWA with the addition of 15.38 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ l⁻¹ and 15.38 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ l⁻¹ and in seawater broth (SWB) supplemented

with 1.8 mM (final concentration) decoyinine, psicofurane or psicofurane tetraacetate (Zain-ul-Abidin *et al.*, 1983; Rüger & Tan, 1992). Evaluation of spore formation was done microscopically after 4 and 8 weeks incubation at 4 °C. The composition of SWB is given below.

Physiological characterization of strains. The tests for identification of strains were performed in seawater media as described previously (Rüger & Richter, 1979; Rüger & Höfle, 1992). Test temperatures were 4 °C; only the 20 °C isolates A03029, A03051 and A03126 were tested at 20 °C. Fermentation of carbohydrates was determined with the Minitek identification system after 2, 4, 7 and 14 d incubation (Rüger, 1981). Fermentation of glucose was additionally tested in the marine oxidation/fermentation (MOF) medium developed by Leifson (1963).

Susceptibility to antibiotics was determined with Oxoid disks loaded with the various amounts of antibiotics given, placed on agar plates prepared with 10 ml SWA.

Utilization of organic compounds as sole sources of carbon and energy was studied in standard microtitre plates by means of a multipoint inoculator (Rüger, 1988). In order to detect possible cross-contamination, three neighbouring wells were taken for each strain. Turbidity was used as an indicator for substrate utilization and was measured with a microtitre plate photometer at 405 nm after 2, 4, 6 and 8 weeks incubation. An increase in turbidity of more than 0.1 OD unit was considered a positive result.

The composition of the cell walls, i.e. the presence/absence of diaminopimelic acid (DAP), was evaluated following the method described by Kandler & Weiss (1986).

Growth characteristics of strains. Optimal and maximal growth temperatures of the tropical isolates were determined at 4, 12, 18, 24, 30 and 37 °C in SWB, consisting of 5.0 g Bacto Peptone, 1.0 g yeast extract, 0.01 g $\text{FePO}_4 \cdot 4\text{H}_2\text{O}$, 750 ml seawater and 250 ml distilled water; pH 7.6. Growth was measured by monitoring optical density at 650 nm with a Gilford photometer model 250. For the polar isolates, the microtitre plate method as described above was used with the same seawater medium, but with incubation temperatures of 1, 4, 8, 12, 18, 24, 30 and 37 °C for the Arctic strains and 1, 4, 8, 12, 16, 20, 24, 30 and 37 °C for the Antarctic strains. Turbidity measurements were performed after 3, 7 and 14 d incubation and turbidity increases of > 0.1 OD unit were taken as positive results.

The microtitre plate method was also applied to determine the NaCl requirements of the strains. The basic test medium, supplemented with 1 ml of a vitamin stock solution l⁻¹ (Rüger, 1988), has been described previously (Rüger & Hentzschel, 1980). Growth was tested at the following NaCl concentrations: 0, 25, 100, 200, 300, 400, 600, 800, 1000, 1500, 2000, 2500 and 3000 mM. Turbidity was measured after 1, 3, 7, 14 and 21 d incubation at 4 and 20 °C.

Seawater requirements of the strains were tested by comparing growth in SWB and freshwater broth (FWB), containing the same constituents as SWB but prepared with distilled water.

Interpretation of the test results for growth temperatures and NaCl requirements. A maximum growth temperature of 24 °C, for instance, means that growth occurred at 24 °C but not at the next higher temperature tested. Thus, the real maximum is somewhere between 24 and 30 °C. Similarly, a

Table 1. Strains used in this study

Agar plates for isolation of strains were incubated at 2 °C (tropical isolates) or 4 °C (polar isolates). Exceptions were strains A03029, A03051 and A03126, isolated from agar plates incubated at 20 °C. Strains were isolated on the following expeditions: GEOTROPEX '83, 1983 (tropical Atlantic), ARK IV/2, 1987 (Arctic Ocean) and ANT VIII/6, 1990 (Antarctic Ocean).

Strain	Sampling site			Depth (m)	Sediment layer (cm)
	Station	Latitude	Longitude		
Tropical Atlantic					
A02605 (= DSM 12258)	373	09° 14-3′ N	19° 37-7′ W	4694	4–6
A02685 (= DSM 12323), A02712, A02720, A02735, A02736, A02738	381	05° 37-0′ N	19° 58-9′ W	2811	0–2
A02761, A02766, A02767 (= DSM 11891), A02788, A02800	381	05° 37-0′ N	19° 58-9′ W	2811	4–6
A02819 (= DSM 11894)	383	02° 59-8′ N	22° 02-5′ W	4514	0–2
A02827	388	16° 56-2′ N	17° 55-5′ W	2766	0–2
A03029 (= DSM 11898)	371	09° 55-3′ N	17° 52-8′ W	1507	4–6
A03051 (= DSM 11899)	373	09° 14-3′ N	19° 37-7′ W	4694	4–6
A03126 (= DSM 12259)	376	09° 08-2′ N	19° 01-8′ W	4802	4–6
Arctic Ocean					
AR3353 (= DSM 12296)	11/223	75° 33-3′ N	08° 48-8′ W	2629	0–2
AR3393 (= DSM 11895), AR3396 (= DSM 11892), AR3398 (= DSM 11893)	11/235	75° 09-4′ N	12° 27-6′ W	403	0–2
AR3447	11/248	72° 13-4′ N	16° 04-3′ W	1450	0–2
Antarctic Ocean					
GU4214 (= DSM 12359)	16/516	66° 03-7′ S	33° 16-6′ E	1357	0–2
GU4223 (= DSM 12356), GU4227, GU4233 (= DSM 11896), GU4234 (= DSM 11897)	16/518	64° 57-3′ S	33° 37-7′ E	2225	0–2
Reference strain					
<i>B. marinus</i> DSM 1297 ^T (= ATCC 29841 ^T)					

maximum NaCl concentration of 1000 mM means growth at 1000 mM but not at the next higher concentration tested, and a minimum of 100 mM means growth at 100 mM but not at the next lower concentration, of 25 mM.

DNA base composition. The G + C content of the DNA was determined from the thermal denaturation profile (Marmur & Doty, 1962), obtained with a Gilford model 250 spectrophotometer equipped with a Gilford model 2527 thermo-programmer and calculated by using the equation of De Ley (1970). DNA from *Escherichia coli* K-12 was used as a reference. G + C ratios were determined from nine melting curves per strain.

DNA-DNA hybridization. DNA-DNA relatedness was determined from renaturation rates according to the protocol given by Fritze *et al.* (1990), based on the methods reported by De Ley *et al.* (1970) and Gillis *et al.* (1970).

For hybridizations with the type strain of *B. marinus*, DNA preparation was done according to Cashion *et al.* (1977). Renaturation rates of these analyses were calculated using the TRANSFER.BAS computer program of Jahnke (1992).

16S rDNA sequence determination and analysis. Genomic DNA extraction, PCR-mediated amplification of 16S rDNA and sequencing of the PCR products were as described by Rainey *et al.* (1996). The sequence reaction products were electrophoresed using an Applied Biosystems 373A DNA sequencer. The 16S rDNA sequences were aligned manually with members of *Bacillus* rRNA group 2 using the ae2 editor

(Maidak *et al.*, 1996). Evolutionary distances were calculated by the method of Jukes & Cantor (1969). Phylogenetic dendrograms were constructed using the treeing algorithm of De Soete (1983).

RESULTS

Growth temperatures and NaCl requirements

From tropical deep-sea sediments, 131 *Bacillus* strains were isolated from plates incubated at 2 °C and 147 from plates incubated at 20 °C. In contrast to the latter, the majority of the 2 °C strains required seawater media for growth, were psychrophilic or psychrotolerant (Table 2) and had almost identical phenotypic characteristics (Rüger & Tan, 1992). Twenty-one taxonomically and physiologically comparable *Bacillus* strains were isolated from Arctic sediment samples and 41 from Antarctic sediment samples.

Besides testing the seawater requirements of all 340 isolates by comparing growth in SWB and in FWB, the minimum, optimum and maximum NaCl concentrations for growth were additionally determined for the 27 strains chosen as representative of all strains. The NaCl requirements of the strains in relation to their growth temperatures are compiled in Table 2. All

Table 2. Growth temperatures and NaCl requirements of *B. marinus* strains

All strains were able to grow at the lowest temperatures tested, namely 1 °C for the polar isolates, the isolation temperature of 2 °C for the tropical deep-sea strains and 4 °C for the three 20 °C isolates A03029, A03051 and A03126. NG, No growth.

Strain	Growth temperature (°C)		NaCl requirement (mM) at 4 °C		
	Optimum	Maximum	Minimum	Optimum	Maximum
Tropical Atlantic					
A02605	18–24	24	0	300–400	1000
A02685	4	12	200	300	400
A02712	4	18	100	300–400	800
A02720	4	12	200	200–300	400
A02735	12	18	100	200–400	800
A02736	12	18	200	400	800
A02738	12	18	300	300	600
A02761	4	12	100	400	600
A02766	12	24	200	300	800
A02767	4	4	100	200	400
A02788	12	24	100	300–400	1000
A02800	4	4	100	300	800
A02819	4–12	24	100	200–300	800
A02827	12	24	25	200–300	1000
A03029	12–24	24	25	300–400	1500
A03051	12–24	30	100	300–400	1000
A03126	12–24	24	100	400	1000
Arctic Ocean					
AR3353	4	12	200	300–400	1000
AR3393	8	12	100	300–600	1000
AR3396	4	12	100	300	800
AR3398	1–4	4	200	300–400	1000
AR3447	8–12	12	NG	NG	NG
Antarctic Ocean					
GU4214	8–12	16	100	300–400	1000
GU4223	8	16	100	200–300	600
GU4227	8	12	200	300–400	800
GU4233	8	16	100	200–400	800
GU4234	8–12	20	100	200–400	1000

strains except A02605 required at least 25 or 100 mM NaCl for growth. The optimum concentrations of 200–400 mM correspond to 1.2–2.4% NaCl, approximately the natural NaCl concentration of seawater. In comparison with SWB, the synthetic medium used in the NaCl requirement tests seemed to be suboptimal. Strain A02819, for instance, still showed slight growth at 24 °C in SWB, but failed to grow at 20 °C in the salt test (result not presented in Table 2), and the psychrophilic strain AR3447 did not grow in the salt test even at 4 °C.

Morphological and physiological characterization

Cell morphology and colony form details are given below in the emended description of *B. marinus*. Not all strains were motile; only 12 of the 27 strains formed endospores and in eight other strains, spore formation was questionable (Table 3). Additional sporulation

could not be induced by the use of media containing decoyinine, psicofuranine or psicofuranine tetraacetate.

Biochemical and nutritional characters for which all strains were positive or negative are also given in the emended species description. Characteristics for which there were strain differences are listed in Table 3. Fermentation of carbohydrates was tested with the Minitex system. For glucose, the MOF medium of Leifson (1963) was additionally used. All strains produced acid from glucose in the MOF medium, but only the Arctic and Antarctic isolates gave positive results with the Minitex system. There is no reasonable explanation for the differences in carbohydrate fermentation results obtained with these two methods, since the Minitex system proved to be useful for the differentiation of marine bacteria in a comparative study (Rüger, 1981). The polar strains seem to be more active than the tropical isolates, as can be seen from the

Table 3. Phenotypic characteristics that differ among *B. marinus* strains

Strains are identified as: Ref, DSM 1297^T; 1, A02605; 2, A02685; 3, A02712; 4, A02720; 5, A02735; 6, A02736; 7, A02738; 8, A02761; 9, A02766; 10, A02767; 11, A02788; 12, A02800; 13, A02819; 14, A02827; 15, A03029; 16, A03051; 17, A03126; 18, AR3353; 19, AR3393; 20, AR3396; 21, AR3398; 22, AR3447; 23, GU4214; 24, GU4223; 25, GU4227; 26, GU4233; and 27, GU4234. Characteristics are scored as: +, positive; (+), weakly positive; (−), very weak positive reaction; −, negative; ?, formation of endospores questionable; NT, not tested. Characteristics of strain DSM 1297^T, the type strain of *B. marinus*, were taken from Rüger & Richter (1979) and Rüger (1983).

Characteristic	Tropical Atlantic																	Arctic Ocean					Antarctic Ocean						
	Ref	2 °C Isolates													20 °C Isolates				18	19	20	21	22	23	24	25	26	27	
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17											
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NT	−	+	−	+	+	+	+	−	−	−	+
Spore formation	+	?	?	+	?	?	?	+	?	+	−	+	−	+	?	+	+	+	+	+	+	+	−	−	−	−	−	−	+
Oxidase (Kovacs)	(+)	+	(+)	(+)	+	(−)	+	+	(+)	(+)	(+)	+	+	(+)	+	+	(−)	+	+	+	+	+	+	+	+	+	+	+	+
Indophenol oxidase	−	−	−	−	(+)	−	−	−	(−)	(−)	−	(−)	−	−	−	+	(+)	(−)	(−)	(−)	(−)	(−)	−	(−)	(−)	(−)	(−)	(−)	(−)
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	(+)	(+)	(−)	(−)	+	+	+	+	+	+
β-Galactosidase (ONPG method)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NT	NT	NT	NT	NT	NT	NT	NT	−	+	+	(+)	+	+
β-Glucosidase	NT	(+)	+	−	−	(+)	+	+	(+)	+	+	+	+	+	+	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
DNase	NT	(−)	(+)	−	−	+	+	(−)	+	+	(+)	+	+	+	+	+	+	+	−	−	(+)	(−)	−	−	−	−	−	−	−
Lecithinase	NT	−	−	−	−	−	−	−	−	−	−	−	−	−	−	(+)	−	−	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
Urease	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	(−)	(+)	−	(−)	−	−	−	(+)	−	−	−	−	−	−
Aesculin hydrolysis	+	−	−	−	−	−	+	(+)	−	−	−	−	−	−	(−)	(−)	(+)	+	+	+	+	−	+	+	+	+	+	+	+
Nitrate reduction to nitrite	−	(+)	−	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	+	−	−	−	−	−	−
Gelatin hydrolysis	+	+	−	+	−	+	(−)	(−)	+	(−)	−	+	−	+	(−)	+	+	+	+	+	+	+	+	+	−	−	−	+	+
Casein hydrolysis	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
H ₂ S production from cysteine	+	+	(+)	−	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	(+)	(+)	(−)	(−)	−	(+)	(−)	(+)	(+)	(+)
Susceptibility to:																													
Pteridine 0/129, 10 µg	+	+	+	+	+	−	−	+	+	−	+	−	+	+	+	(+)	−	+	+	+	+	+	+	+	−	+	+	+	+
Acid from (Minitex system):																													
Glucose	+	(−)	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	−	−	−	−	−	−	−	−	−	−	−	−	−	(−)	+	−	−	(+)	+	(+)	(−)	(−)	+	(+)	(+)	(+)	(+)	+
Maltose	+	−	−	−	−	−	−	−	−	−	−	−	−	−	(+)	+	−	−	−	−	−	−	(+)	−	−	−	−	−	−
Mannose	+	(−)	(−)	−	−	(−)	−	−	−	−	−	−	−	−	(+)	−	(+)	+	+	+	+	+	+	+	+	+	+	+	+
Trehalose	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	(+)	−	−	−	−	−	−	−	−	−	−	−	−
Xylose	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−

oxidase, β-galactosidase, aesculin, gelatin and mannose test results.

Presence/absence of DAP

One-dimensional TLC of whole-cell lysates revealed the absence of DAP from the cell walls of the representative strain A02819 (=DSM 11894). Previous analysis of the type strain, *B. marinus* DSM 1297^T, had shown that the peptide side chains of the peptidoglycan are linked directly through L-lysine, which carries a glycine bound to the carboxyl group (N. Weiß, personal communication).

DNA base composition and DNA–DNA homology

The DNA base composition was determined for 16 strains and was in the range of 39.6 to 41.6 mol% G + C. No differences were found between the strains of the three sampling sites.

DNA–DNA homologies between strains from all three geographical regions are shown in Table 4. Most homology values were greater than 70% and showed clearly that the strains from the tropical deep sea and the Arctic and Antarctic oceans belonged to a single species. The high 75% DNA–DNA homology value of strain A02819 with the type strain of *B. marinus*, strain

DSM 1297^T, verified the new isolates as members of this species.

Furthermore, high DNA–DNA homology values between the spore-forming isolates and the three non-spore-forming strains A02767, A02800 and GU4233 confirmed that the non-spore-formers were bacilli that had lost the ability to form endospores.

16S rDNA sequence determination

Eight strains (A02767, A02819, A03029, A03051, AR3393, AR3396, AR3398, GU4234) were selected for partial 16S rDNA gene sequence analysis, including strains from all three expeditions. All strains clustered within *Bacillus* rRNA group 2 and were found to be closest to *B. marinus* DSM 1297^T. The 16S rDNA gene sequence similarity values to this strain were in the range of 98.7 to 100.0%. The closest phylogenetic neighbour is *B. insolitus*, with a similarity value of 97.1%.

Phylogenetic dendrograms constructed by using the treeing algorithm of De Soete (1983) are not shown here, but are available on request.

Strain A02819 was chosen for complete 16S rDNA gene sequence analysis because of its lowest 16S rDNA gene sequence similarity compared with the sequence

Table 4. DNA–DNA homology values between pairs of *B. marinus* strains

Values are percentage DNA homology. The type strain of *B. marinus*, DSM 1297^T, is identified as Ref. –, Not determined.

Strain	Ref	Tropical Atlantic					Arctic		Antarctic	
		1	2	3	4	5	6	7	8	9
1. A02767	–	101								
2. A02800	–	65	99							
3. A02819	75	67	71	101						
4. A03029	–	69	67	80	101					
5. A03051	–	–	68	74	79	100				
6. AR3396	–	–	–	81	77	78	100			
7. AR3398	–	70	67	78	70	75	81	100		
8. GU4233	–	–	69	82	77	76	83	77	101	
9. GU4234	–	69	70	84	72	75	82	82	83	102

of *B. marinus*. On this basis, the similarity value rose to 99.4%.

DISCUSSION

Characteristics and taxonomy of the strains

On the basis of phenotypic characteristics (Table 3), the absence of DAP, DNA base composition, DNA–DNA homology (Table 4) and partial and complete 16S rDNA gene sequence analysis, the new isolates were assigned to the obligately marine species *B. marinus*.

At the time of the first isolations of *B. marinus* strains from the Iberian deep sea, tests for utilization of organic compounds as sole sources of carbon and energy were not generally applied. As a consequence, the original descriptions of the species (Rüger & Richter, 1979; Rüger, 1983) must be regarded as incomplete compared with the current standard. Therefore, combining the taxonomic characteristics of the 18 *B. marinus* strains already described (Rüger & Richter, 1979; Rüger, 1983) and the new isolates, an emended description of the species is presented below.

Distribution and origin of *B. marinus*

The formation of endospores as a survival strategy in terrestrial habitats is an important taxonomic characteristic of the genus *Bacillus*. However, the ability to form endospores may not be necessary in environments with long periods of uniform environmental conditions, like the deep sea. This may explain why endospores could be detected in only a few of the new *B. marinus* isolates.

Strains of *B. marinus* have been isolated from sediments of the Iberian deep sea, the tropical Atlantic and the Arctic and Antarctic Oceans. It is not likely that a bacterial species could evolve simultaneously in such distant places. Taking into consideration the known

deep-water circulation in the polar and tropical sectors of the Atlantic Ocean (Mantyla & Reid, 1983; Hollister & McCave, 1984), it is assumed that the origin of *B. marinus* was in the Antarctic and that the species spread from there to the north.

Emended description of *Bacillus marinus* (Rüger and Richter) Rüger 1983, emend. Rüger, Fritze and Spröer

Bacillus marinus (ma'rin.us. L. n. *marinus* marine).

Gram-positive rods, 0.8–1.1 µm in diameter and 2.0–7.0 µm in length. Filaments present. Occurring singly, in pairs and short chains. Motile strains are peritrichously flagellated or the peritrichous flagellation is degenerate. Endospores are round or slightly ellipsoidal and are located terminally to subterminally. The sporangia are not or are only slightly swollen. Strains without the ability to form endospores have been isolated. Colonies on seawater agar are round with entire margin, flat to raised, colourless, mostly translucent; diameter 1–2 mm after 2 weeks incubation (the extremely psychrophilic strains require longer incubation times of up to 4 weeks at 4 °C). Strains require seawater media for growth; the optimum NaCl concentration for growth is 200–400 mM, i.e. 1.2–2.4%. Growth occurs at 1–4 °C; strains are psychrophilic or psychrotolerant. The highest maximum growth temperature found was 30 °C and numerous strains did not grow at temperatures exceeding 4 or 8 °C.

The following characteristics are positive for all strains tested: catalase, aerobic acid production from glucose in MOF medium, susceptibility of the 27 new strains to 150 µg pteridine 0/129 (2,4-diamino-6,7-diisopropyl pteridine phosphate), 10 µg chloramphenicol and 50 µg furazolidone (the 18 previous isolates have not been tested).

The following characteristics are negative for all strains tested: anaerobic growth, indole, methyl red, Voges–Proskauer, arginine dihydrolase, lysine decarboxylase,

Table 5. Differentiation of cold-adapted *Bacillus* and *Paenibacillus* species

Data without superscripts were obtained in the present study. The remaining data were obtained from the following references, as indicated by superscripts: 1, Stackebrandt *et al.* (1987); 2, N. Weiß (personal communication); 3, Fahmy *et al.* (1985); 4, F. G. Priest (personal communication); 5, Ash *et al.* (1991); 6, Nakamura (1984); 7, Rüger (1983); 8, Gordon *et al.* (1973); 9, Claus & Berkeley (1986); 10, Abd El-Rahman (1988); 11, Larkin & Stokes (1967); and 12, Rüger & Richter (1979). Characteristics are scored as: +, positive; −, negative; ±, variable result; v, varies among strains. Bd, Buoyant density.

Property	<i>B. marinus</i>	<i>B. globisporus</i>	<i>B. insolitus</i>	<i>P. macquariensis</i>	<i>B. psychrophilus</i>	<i>B. psychrosaccharolyticus</i>
Spore shape	Round-ellipsoid	Round ¹²	Round-cylindrical ¹²	Ellipsoid ⁹	Round ¹²	Ellipsoid ¹²
Sporangium shape	Not swollen	Swollen ¹¹	Not swollen ¹¹	Swollen ⁹	Swollen ¹¹	Swollen ¹¹
Minimum growth temperature (°C)	<1–4	<0 ¹¹ , −2 ¹⁰ , 3 ⁸	<0 ¹² , −2 ¹⁰ , 3–5 ⁸	0 ¹¹ , −2 ¹⁰	<0 ¹¹ , −2–5 ¹⁰ , 3 ⁸	0 ¹¹ , −2 ¹⁰ , 3 ⁸
Growth at:						
23/24 °C	v	+ ¹¹	+ ¹¹	+ ¹⁰	+ ¹¹	+ ¹¹
30 °C	v	− ¹¹	− ¹¹	− ^{9,10}	+ ¹¹	+ ¹¹
NaCl required	+	− ⁹	− ⁹	− ⁹	− ¹¹	− ¹¹
Growth at:						
2% NaCl	+	+ ¹¹	± ¹¹	+ ⁹	+ ¹¹	+ ¹¹
3% NaCl	+	− ⁶ , + ¹⁰	− ¹⁰	− ⁹	+ ^{6,10}	+ ¹⁰
5% NaCl	v	− ^{9,10}	− ^{9,10}	− ^{9,10}	+ ^{6,10}	− ¹⁰
Anaerobic growth	−	− ¹⁰ , + ¹¹	− ^{10,11}	+ ^{9,10}	+ ^{6,11} , − ⁸	+ ^{8,11} , ± ¹⁰
Starch hydrolysis	−	− ^{6,11}	− ¹¹	+ ^{9,10}	− ^{6,11}	+ ¹¹
Urea digestion	−	+ ¹¹	− ¹¹	− ^{9,10}	+ ^{6,11}	− ¹¹
NO ₃ reduction to NO ₂	v	− ^{6,11}	− ¹¹	− ^{9,10}	+ ^{6,11}	+ ¹¹
Cell wall	L-Lys direct (Gly) at carboxyl group ²	L-Lys-D-Glu ¹	L-Orn-D-Glu ¹	DAP ¹⁰	L-Lys-D-Glu ¹	DAP ¹⁰
DNA G + C content (mol %)	39.6–41.6 (<i>T_m</i>), 36.9–39.5 (<i>T_m</i>) ¹² , 37.6 (<i>T_m</i>) ³ , 38.0 (Bd) ³	39.8 (<i>T_m</i>) ³ , 39.7 (Bd) ³ , 40.6 (<i>T_m</i>) ⁷	35.9 (<i>T_m</i>) ³ , 36.1 (Bd) ³	39.3 (<i>T_m</i>) ³ , 41.6 (Bd) ³	39.7 (<i>T_m</i>) ^{3,7} , 40.5 (Bd) ³ , 44.1 (Bd) ⁹	43–44 ⁴
rRNA Group	2	2 ⁵	2 ⁵	3 ⁵	2 ⁵	1 ⁵

ornithine decarboxylase, nitrate reduction to gas and digestion of starch and chitin.

No aerobic acid production (Minitek system) from arabinose, cellobiose, lactose, mannitol, raffinose, rhamnose, salicin, sorbitol or sucrose. The following compounds are not utilized. Carbohydrates: L-arabinose, cellobiose, D-galactose, gluconate, D-glucose, maltose, D-mannose, D-ribose, salicin, sucrose, trehalose and D-xylose. Carboxylic acids: acetate, adipate, citrate, DL-3-hydroxybutyrate, fumarate, lactate, propionate, pyruvate and succinate. Alcohols: glycerol, mannitol and sorbitol. Amino acids: L-alanine, L-arginine, L-aspartate, L-glutamate, L-histidine, L-lysine and L-ornithine. Others: putrescine, *p*-hydroxybenzoate and quinate.

The following characteristics give strain-dependent results: oxidase (Kovacs), indophenol oxidase, β-galactosidase, β-glucosidase, DNase, lecithinase, urease, aesculin hydrolysis, NO₃ reduction to NO₂, gelatin hydrolysis, casein hydrolysis, H₂S from cysteine, susceptibility to 2 U penicillin G, 10 µg pteridine 0/129 and 10 µg tetracycline. Acid from the following carbohydrates, determined with the Minitek system: glucose, glycerol, maltose, mannose, trehalose and xylose.

The G + C content of DNA (*T_m*), based on 16 of the new isolates, is 39.6–41.6 mol%. The G + C contents of four of the previous isolates from the Iberian deep sea were in a lower range of between 36.9 and 39.5 mol%. Results of DNA G + C content analyses of the type strain: 39.3 [Rüger & Richter, 1979 (*T_m*)],

37.6 [Fahmy *et al.*, 1985 (*T_m*)] and 38.0 mol % [Fahmy *et al.*, 1985 (buoyant density)]. The EMBL accession number of the 16S rDNA gene sequence of *B. marinus* DSM 1297^T is AJ237708.

The type strain is DSM 1297^T (=ATCC 29841^T). Source: isolated from sediments of the Iberian deep sea, the tropical eastern Atlantic and the Arctic and Antarctic seas. Habitat: Marine sediments. Characteristics useful for differentiation of *B. marinus* from other psychrophilic or psychrotolerant *Bacillus* and *Paenibacillus* species are listed in Table 5.

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